The Role of IL-18 and IL-18Rα in the Development of Autoimmunity

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DISCLAIMER

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1. Gutcher I., Urich E., Wolter K., Prinz M. & Becher B. (2006). IL-18-independent engagement of IL-18Rα is required for the development of autoimmune inflammation. Nat. Immunol., 7(9): 946-953

2. Gutcher I. & Becher B. (2007). APC-derived cytokines and T cell polarization during autoimmune inflammation. J. Clin. Invest., 117(5):1119-1127

CONTENTS

SUMMARY

Organ-specific autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, are driven by the conduct of auto-aggressive T helper cells. It has long been accepted that T_H1 cells are the major pathogenic cell population in autoimmune inflammation while T_H2 cells are beneficial in what is known as the T_H1/T_H2 paradigm. However, recent data obtained in gene-targeted mice and the newly discovered T_H17 phenotype have proved conflictive with this hypothesis and have provoked a paradigm shift. It is thus becoming widely accepted that T_H1 cytokines and T_H1-inducing cytokines are not pathogenic and may actually have protective roles in autoimmune inflammation. Instead, the novel population of IL-17-secreting $CD4^+$ T cells has replaced the T_H1 cell subset as the major effector population in autoimmunity.

The polarization of effector $CD4^+$ T cells relies critically upon the actions of cytokines secreted by antigen-presenting cells (APCs). IL-18 is a vital cofactor, together with IL-12, in promoting T_H1 polarisation and IFNγ secretion. Similar to other T_H1 cytokines, IL-12 is no longer considered to be a pathogenic molecule in the context of autoimmune inflammation. We therefore decided to examine the role of IL-18 and its receptor in the animal model of autoimmune CNS-inflammation, Experimental Autoimmune Encephalomyelitis (EAE). As with IL-12-deficient mice, we found that IL-18-deficient mice are fully susceptible to EAE. Surprisingly, we discovered that mice deficient in IL-18Rα are EAE-resistant, suggesting the presence of an alternative ligand with encephalitogenic properties. The actions of this alternative ligand could be blocked *in vivo* using a monoclonal antibody against IL-18Rα, which resulted in the abrogation of EAE development. Furthermore, our results establish that IL-18 and T_H1 development are dispensable for autoimmune inflammation, while IL-18Rα engagement on APCs is essential for IL-23 secretion and the subsequent generation of encephalitogenic T_H17 cells.

ZUSAMMENFASSUNG

Organ-spezifische Autoimmunerkrankungen wie Multiple Sklerose oder Rheuma werden von autoaggressiven T Helfer (T_H) Zellen verursacht. Lange wurde von dem sogenannten T_H1/T_H2 Paradigma ausgegangen, dem zufolge T_H1 Zellen die pathogene T_H-Zellpopulation darstellen, wohingegen T_H2-Zellen eine förderliche Rolle im Krankheitsverlauf spielen. Ergebnisse aus Studien mit genveränderten Mäusen und die kürzlich entdeckten T_H17 Zellen stehen im Widerspruch zu dieser Hypothese und haben einen Paradigmenwechsel ausgelöst. Man geht nun davon aus, dass T_H1 Zytokine und T_H1 induzierte Zytokine nicht pathogen sind, sondern eventuell sogar eine positive Rolle in autoimmunen Entzündungen spielen. Es gelten nicht mehr T_H1- sondern die unlängst beschriebenen IL-17 sekretierenden $CD4^+$ T Zellen als die Haupteffektoren der Autoimmunität.

Die Polarisierung von Effektor- $CD4^+$ T Zellen beruht vor allem auf der Wirkung von Zytokinen, die von Antigen-präsentierenden Zellen (APCs) sekretiert werden. IL-18 ist, zusammen mit IL-12, ein entscheidender Cofaktor für die Polarisierung von T_H1 Zellen und die IFNγ Sekretion. Im Kontext autoimmuner Entzündungen gilt IL-12, wie auch andere T_H1 Zytokine, nicht mehr als pathogenes Molekül. Daher wollten wir die Rolle von IL-18 und dessen entsprechendem Rezeptor IL-18R in Experimenteller Autoimmuner Enzephalomyelitis (EAE), einem Tiermodell der autoimmunen Entzündung im ZNS, untersuchen. Wir konnten beobachten, dass sich der Krankheitsverlauf in IL-18 defizienten Mäusen nicht von dem in wt Mäusen unterschied – gleiches wurde bereits für IL-12 defiziente Mäuse gezeigt. Überraschenderweise fanden wir, dass IL-18R defiziente Mäuse vollständig resistent gegen EAE sind, was auf die Existenz eines alternativen Liganden mit enzephalitogenen Eigenschaften für den IL-18R schliessen lässt. Die Wirkung dieses Liganden konnte *in vivo* durch einen monoklonalen Antikörper gegen den IL-18R blockiert werden, wodurch die Entwicklung von EAE abgeschwächt werden konnte. Ausserdem zeigen unsere Ergebnisse, dass IL-18 und die T_H1-Entwicklung für die Entstehung von autoimmunen Entzündungen

erlässlich sind, während die Stimulierung des IL-18R auf APCs für die Sekretion von IL-23 und der folgenden Bildung von enzephalitogenen T_H17 Zellen essentiell ist.

GENERAL INTRODUCTION

AUTOIMMUNE INFLAMMATORY DISEASES

Autoimmune diseases occur when the complex network of immune cells, that usually act to protect the body from pathogenic invasion, initiates an attack against the body's own tissues and organs. The recognition of foreign antigen (Ag) relies critically on the random generation of Ag receptors on the surface of lymphocytes. This recombination event yields a high number of Ag receptors, some of which are potentially self-reactive. The process of central tolerance is designed to recognise and eliminate self-Ag reactive, and therefore possibly harmful, lymphocytes. However the healthy immune system does contain circulating self-reactive lymphocytes that have escaped central tolerance and mechanisms of peripheral tolerance exist that operate to control and suppress an outright autoimmune attack by these cells (1). Under certain conditions, however, which mostly remain unclear, there is a loss of peripheral tolerance to self-Ags that leads to the inappropriate expansion of self-reactive effector cells and ensuing tissue inflammation. Autoimmune inflammatory diseases fall into two categories: systemic and tissue-specific. Systemic autoimmune diseases, such as systemic lupus erythematosus (SLE), affect a variety of organs and mostly result from a humoral immune response. On the other hand, tissue-specific autoimmune diseases target one organ only. For example, the target tissues of the diseases multiple sclerosis (MS), rheumatoid arthritis (RA) and type I diabetes are the central nervous system (CNS), the joint synovium and the beta cells of the pancreas, respectively. Furthermore, and in contrast to systemic autoimmune inflammation, tissue-specific autoimmune inflammation is generally held to be cell-mediated. Auto-reactive T helper cell type 1 (T_H1) lymphocytes have long been associated with such autoimmune diseases although more recent evidence suggests a role for the newly discovered T_H17 cells. Despite a central role for T_H cells, Ag-presenting cells

(APC) are also key players during both the initiation and progression of the autoimmune response. APCs perform multifarious tasks in the peripheral immune compartment and, importantly, generate guidance cues directing T cell polarization. Consistently, during an autoimmune reaction, the development of auto-reactive T cells into pathogenic and destructive effector cells relies critically upon the secretion of soluble cytokines by APCs. Thus the capacity of APC-derived cytokines to polarise T cells is of significant importance as it endows APCs with the potential to either promote or suppress the development of autoimmune disease. This thesis presents evidence demonstrating the role for the APC-derived cytokine IL-18 and its receptor IL-18Rα in the development of auto-reactive T_H cells and does so in the context of CNS autoimmune inflammation.

CNS AUTOIMMUNE INFLAMMATION

The CNS has long been considered an organ that is inaccessible to immune cells and hence has been referred to as an "immune privileged site". This hypothesis was based on the evidence that grafts and tumours fare much better in the CNS and was mainly attributed to the separation of the CNS from the systemic and lymphatic compartments (2-4). The blood-brain barrier (BBB) is a highly impermeable capillary endothelium that is formed from specialized endothelial cells that are in contact with the endfeet of astrocytes and pericytes (5;6). The endothelium is held tightly together by tight junctions, which occludes leukocyte infiltration into the CNS, and in contrast to other vasculature, demonstrates little vesicular transport activity (7;8). Furthermore, the absence of draining lymphatics from the CNS led to the assumption that T cells would never be exposed to CNS Ag (3). Despite the lack of communication between the CNS and the periphery, there is mounting evidence that the CNS is not as privileged as once thought. Not only can activated lymphocytes readily traffic into the CNS through post-capillary venules (9) but CNS Ag is able to drain into the cervical lymph nodes (LN) (10), while cells residing within the CNS are themselves capable of

presenting Ag (11). Furthermore, extensive inflammatory cell infiltration occurs during CNS autoimmune inflammation, which is described below.

CNS Autoimmune Inflammation in Humans

MS is an inflammatory and demyelinating disease of the CNS during which immune-mediated responses are conducted against specific CNS Ag, namely myelin proteins, which are present in the protective myelin sheath surrounding neuronal axons. The myelin sheath is responsible for the fast conduction of electric impulses along neuronal fibres and its destruction results in the characteristic symptoms of MS including sensory and visual disturbances, paralysis and lack of coordination (12). The pathological features of the MS CNS include distinct foci of inflammatory lesions, containing lymphocytes and monocytes, with evidence of demyelination and axonal loss, as well as BBB leakage and astrogliosis (13). MS, which follows a recurrent or chronic time-course, develops spontaneously in young adults and has a prevalence of 1/1000 in Western Europe. Although the aetiology of the disease remains unclear, both genetic and environmental factors have been postulated (14). The genetic contribution to MS susceptibility is demonstrated in monozygotic twins, whereby there is a 30% concordance in the occurrence of the disease (15). The only major gene locus associated with the disease is the human leukocyte antigen (HLA) region, whose products encode restriction elements of T cells. An association has been demonstrated between the alleles *HLA-DR1501* and *HLA-DQ0601* and a 2-4 fold increased risk in developing MS in white populations (16). The finding of linkage with the HLA allele has strengthened the argument that MS pathogenesis does have an autoimmune basis. More conclusive evidence though, has been obtained through the use of the animal model for MS, which is known as Experimental Autoimmune Encephalomyelitis (EAE).

CNS Autoimmune Inflammation in Animals

The study of MS pathogenesis and the clarification of the events precipitating the disease have been aided greatly by the development of the EAE model. EAE can be induced in susceptible rodents or monkeys by immunisation with myelin Ag, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP) (17). Similar to MS, the pathology of this neuroparalytic disorder is characterised by lymphocytic and monocytic perivascular infiltration, an increase in BBB permeability, astrocytic hypertrophy and demyelination (18;19). In fact, with exception to the mode of induction, the steps involved in EAE pathogenesis are thought to parallel those of MS. Similar to other organ-specific autoimmune diseases, such as RA and type I diabetes, the pathogenesis of CNS autoimmune inflammation is generally held to be cell-mediated, initiated predominantly by $CD4^+$ T cells. Furthermore, the immunopathological events can be divided into two major phases: an initial T cell priming/activation phase and a subsequent recruitment and effector phase (20). T cell priming is initiated in the secondary lymphoid organs where professional APCs, such as macrophages and dendritic cells (DC), present Ag to neuroAg-reactive T cells leading to their activation and expansion. Prior to initiating tissue damage, activated myelin-reactive T cells that have migrated to the CNS must first re-encounter their cognate Ag. It has been demonstrated that DCs present in the perivascular space of the BBB are responsible for presenting Ag to and reactivating activated lymphocytes, which are then capable of penetrating deeper into the parenchyma (11). Despite a clear role for the immune system in both these events, CNS resident cells play their own role in the effector phase of the disease through the generation and maintenance of the inflammatory milieu. Upon damage to CNS tissue, the resident macrophages of the CNS, known as microglial cells, upregulate major histocompatability complex (MHC) and costimulatory molecules, and secrete cytokines and chemokines (21;22). This promotes the continued infiltration of inflammatory cells into the CNS, while providing ongoing activation for cells already present in the inflammatory

lesion. Certain CNS-derived factors, however, aim to suppress the inflammatory response (22). Therefore the CNS micro-environment also has a critical function in the ongoing autoimmune attack, which is performed in part by microglia-derived cytokines.

PRINCIPLES OF CELL-MEDIATED IMMUNITY

Clearly, cell-mediated events of the immune system are critical to the development of MS and EAE and autoimmune diseases in general. Therefore I will give a brief overview of the workings of the immune system that are relevant to the discussion of the role of APC-derived cytokines in EAE pathogenesis. The immune system is divided into two branches, termed the innate and acquired immune systems, which fight to protect the host from invading pathogens and foreign molecules (23). These branches are not mutually exclusive and in fact they work in concert to defend the organism in both a rapid and specific manner. Innate immunity is responsible for the first rapid response to Ag and does so at the expense of specificity and therefore in a generic fashion. Acquired or adaptive immunity, on the other hand, is specific and can generate immunological memory. A more detailed analysis of the cells and processes involved in these separate yet interacting arms of immunity are described in the following sections.

Innate Immunity

Cells of the innate immune system include NK cells, polymorphonuclear macrophages (PMNs), monocytes/macrophages and DCs. To defend the host against foreign molecules, these cells recognise specific molecular components of invading micro-organisms known as pattern-associated molecular patterns (PAMP). Essential features of PAMPs are that they are absent in the host and are indispensable components of the invading pathogen allowing discrimination between self and non-self and ruling out the possibility of mutation and escape by the pathogen (24). PAMPs are recognised by both soluble and membrane-bound mediators

of innate immune cells via germ-line encoded receptors called pattern recognition receptors (PRR). Soluble mediators include mannose-binding lectins (MBL) (25), C-reactive protein (CRP) (26) and complement (27). Membrane bound receptors, which are usually present on macrophages and DCs, include the scavenger receptors (28) and most importantly the toll-like receptors (TLR) (29). TLRs are the key structures of recognition in the innate immune system permitting it to detect a broad range of pathogens including bacteria, viruses, fungi and protozoa. To data, 12 TLRs have been identified in mouse and 11 in humans. Stimulation of TLRs results in increased phagocytic activity, release of inflammatory mediators and production of reactive oxygen species and secretion of cytokines. As will be discussed later, the pattern of cytokines secreted during the innate immune response crucially affects the development of the subsequent adaptive immune response.

Adaptive Immunity

The cells of the adaptive immune system are known as B and T lymphocytes and they have both high specificity and diversity. These features arise from the ability of the genes encoding the B cell receptor (BCR) and T cell receptor (TCR) to undergo genetic recombination. In addition, B and T lymphocytes have immunological memory, which allows them to mount a faster response, with greater reactivity, upon subsequent recognition. The high recombinatorial capacity of these receptors means that, during development, there is generation of self-reactive receptors. The mechanism of central tolerance aims to ensure the deletion of such self-recognising lymphocytes. Nevertheless, this process isn't completely effective and results in the release of potentially auto-reactive lymphocytes into the circulation. As organ-specific autoimmune diseases are predominantly T cell mediated, I will briefly describe the processes that prevent the development of self-recognition in the context of T cells.

T cells are generated in the bone marrow but must migrate to the thymus for maturation. Upon rearrangement of the alpha and beta chains of the TCR, functional receptors expressed by thymocytes are selected on the basis of two properties during central tolerance (30). The first property is recognition of self-MHC and is known as self-restriction. This is important because, as explained later, T cells can only recognise their Ag in the context of an MHC molecule. Thymocytes that can recognise self-MHC, expressed by thymic epithelial cells, are positively selected for while non-recognition results in apoptosis in a process known as positive-selection. The second property is the ability to recognise foreign Ag. In this process of negative selection, thymocytes with high affinity for self-MHC or recognising self-Ag are eliminated by apoptosis ensuring that there is tolerance to self-Ag. During this process, thymic epithelial cells, as well as specialised DCs, present self-Ag to the developing T cells. This does not pose a problem for ubiquitous Ag but does so for organ-specific Ag, which is unlikely to be expressed in the thymus or, when it is, is often sequestered from thymocytes (1). Tissue-specific Ag can also be thymically expressed, albeit at somewhat lower levels, as a result of transcription factors such as autoimmune regulator (Aire). Indeed CNS-specific Ags, such as MBP, and possibly MOG, have been demonstrated to be expressed in the thymus. Therefore the presence of mature MBP- and MOG-reactive T cells circulating in the periphery is thought to result from specific T cells, with low avidity or recognising particular epitopes, being spared negative selection (1). The mere presence of these auto-aggressive T cells in the periphery, however, does not dispose or increase susceptibility of individuals to autoimmune disease. For example, similar numbers of myelin-reactive $CD4^+$ T cells have been demonstrated in the blood and CSF of MS patients and healthy individuals (31). This implies that mechanisms of peripheral tolerance provide the “brakes” on the possible development of an autoimmune attack.

BRIDGING INNATE AND ADAPTIVE IMMUNITY

Mature T cells are constantly circulating through the blood, lymph and secondary lymphoid organs in search of their cognate Ag. However, even in the presence of its Ag, a precise sequence of events must occur for the T cell to recognise its Ag and become activated. Unlike B cells, T cells cannot directly bind foreign protein or peptide and require help for Ag recognition. Instead, APCs of the innate immune system are required for Ag presentation to T cells and, as such, these cells function to bridge the innate and adaptive arms of immunity. However Ag presentation alone isn't sufficient for an Ag-specific $CD4^+$ T cell to become activated and APCs must deliver three different signals for appropriate T cell activation to occur, as described below and illustrated in figure 1.

Signal 1 – Ag Presentation

The first signal involves the presentation of Ag on the surface of an MHC molecule that allows T cell recognition of their cognate Ag though the TCR (**Fig. I**, **left panel**) (32). $CD8^+$ T cells, which react to extracellular pathogens, recognise their Ag on the surface of a class I MHC molecule. $CD4^+$ T cells, on the other hand, react with foreign peptide on the surface of class II MHC. With regards to $CD4^+$ T cells and exogenous Ag, APCs must initially internalise foreign protein, either by phagocytosis or endocytosis, before proteolytically processing it in the endosomal/lysosomal pathway. Ag is thus degraded into small peptides of 13-18 residues, which can be loaded onto an MHC II molecule and transported to the surface for presentation to the TCR/CD3 complex.

Signal 2 – Costimulation

In order for Ag-specific T cells to become activated and expand, a second signal must be generated through the interaction of adhesion and costimulatory molecules on the surface of APCs and T cells (**Fig. I**, **middle panel**). Indeed, lack of these signals permits maintenance of peripheral tolerance to auto-reactive lymphocytes. Adhesion molecules like ICAM-1 and

LFA-1 strengthen the immunological synapse by causing closer contact between the interacting APC and T cell. Such close interactions are an essential requirement for the successful outcome of signal 3, as described below. Costimulatory signals at the immune synapse are generated by the B7/CD28 family of cell surface molecules (33). The B7 family members are predominantly expressed by APCs. They are either constitutively expressed at low levels and subject to upregulation as in the case of B7-2 (CD86) or inducibly expressed as with B7-1 (CD80). Both these transmembrane ligands interact with CD28 or CTLA-4, another CD28 family member, on the surface of naïve T cells to induce a stimulatory or inhibitory signal, respectively. The potent stimulatory signal they provide to T cells, previously activated through their TCR, not only leads to productive cell activation but it also directs cell expansion and survival as well as growth factor production. Interestingly, CTLA-4 exhibits a much higher affinity for B7 molecules than CD28 does but, unlike CD28, it is not constitutively expressed and undergoes upregulation only upon T cell activation. Thus it functions to attenuate the T cell response. Although I have concentrated here on the APC-derived costimulatory signals necessary for T cell activation, it is important to point out that, in a reciprocal manner, T cells simultaneously provide APCs with stimulatory signals. CD40L (CD154) expressed by T cells interacts with its receptor CD40, which is present on monocytes/macrophages and DCs (34). CD40 signalling has a myriad of effects including cell activation and survival, upregulation of costimulatory molecules such as B7-1 and B7-2 and CD40 itself, and upregulation of adhesion molecules.

Signal 3 – Cytokine Secretion

The third signal delivered by APCs (**Fig. I, right panel**) directs the differentiation of activated Ag-specific lymphocytes into an effector T cell subtype. Although both the affinity of the peptide-Ag-TCR interaction and costimulatory signals influences the differentiation pathway, the secretion of a specific set of cytokines by APCs provides the major signal.

These soluble proteins signal in a paracrine fashion via cytokine receptors explaining the requirement for adhesion molecule interaction to form close contact between APCs and T cells, as described above. The pattern of secreted cytokines depends upon the initial stimulation of APCs by PAMPs. TLR triggering stimulates the secretion of a precise set of cytokines that ultimately results in a specific reaction against that pathogen by the induction of a T_H1 or T_H2 cell response. Therefore the creation of a particular cytokine environment by APCs during immunity is critical for the determination of the appropriate type of immune response, which can be either cell-mediated or humoral. Consistently, during an autoimmune reaction, the development of auto-reactive T cells into pathogenic and destructive effector cells relies critically upon the secretion of soluble cytokines by APCs. It has been demonstrated that the APC-derived proteins IL-12 and IL-18 are key players in the development of a T_H1 cell response and that they synergise with one another by upregulating their reciprocal receptors on the surface of T_H1 cells (35;36). Alternatively, T_H2 cell differentiation occurs in the absence of T_H1-inducing factors and seems to be promoted solely by T cell-derived cytokines, in particular IL-4, subsequent to interaction with APCs (37). The precise actions of individual APC-derived cytokines will be described in more detail below but first I will discuss the differentiation of $CD4^+$ T cell effector types in more depth.

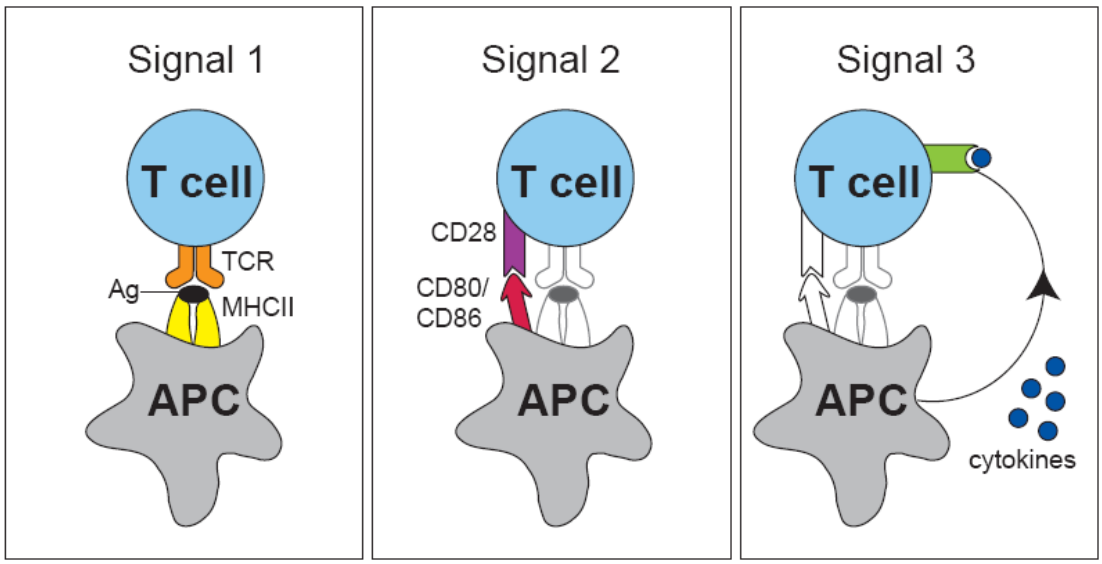


Figure I: T cells require three APC-derived signals for activation. Within the immune synapse that forms between APCs and T cells, three signals are required for Ag-specific $CD4^+$ T cell activation. Signal 1 comprises the presentation of antigen peptide in the context of MHC II molecules, which is recognised by the Ag-specific TCR of the T cell (left panel). Signal 2 involves the stabilisation of the synapse through adhesion molecules and the generation of signals via costimulatory molecules present on the surface of APCs and T cells (middle panel). CD80/CD86 on APCs interacts with their CD28 on T cells to generate activatory signals while interaction with CTLA-4 generates inhibitory signals (not pictured). Signal 3 is produced by the secretion of cytokines from APCs, which signal via cytokine receptors on T cells in order to polarise them towards an effector phenotype (right panel).

T CELL DIFFERENTIATION

Concept of the T_H1 and T_H2 Subset Model

In 1986, Mosmann *et al*. initially proposed a model whereby $CD4^+$ T cells are subdivided into two independent subsets with distinct effector function (38;39). Their hypothesis suggested that T_H cells can be segregated into T_H1 and T_H2 subsets on the basis of cytokine expression and bioactivities as well as helper function. Those and further experiments demonstrated that T_H1 cells predominantly secrete IL-2, IL-3, TNFα and most notably IFNγ and control cell-mediated functions such as the activation of macrophages, while the secretion of IL-4, IL-5

and IL-13 by T_H2 cells leads to the stimulation of humoral immunity by aiding B cell activation and class switch (39). Interestingly, the cytokines of a particular T_H subtype are able to further promote the expansion of that subtype whilst simultaneously cross-regulating and therefore inhibiting the development of the opposite subset. This allows each T_H subset to produce characteristic cytokines that in turn provoke the development of a distinctive effector function specific for that immunogen. Thus, while T_H1 cells induce pro-inflammatory responses, such as delayed type hypersensitivity, and eliminate intracellular infections, T_H2 cells function during allergic reactions and anti-helminth responses. More recently, individual transcription factors have been identified that are essential for the development of T_H1 or T_H2 cells. T-box expressed in T cells (T-bet) is the key transcription factor associated with T_H1 cell development while GATA-binding protein 3 (GATA-3) plays the dominant role in T_H2 differentiation (40;41). Naïve non-differentiated T cells are known as Th0 or T_H precursor (T_Hp) cells and upon their stimulation by Ag, T-bet and GATA-3 are said to be expressed simultaneously at low levels (42). Depending upon the cytokine milieu, either T-bet or GATA-3 is upregulated further. T-bet affects T_H1 differentiation in several major ways: it acts to potentiate IFNγ production, which further promotes T-bet expression in a positive feedback loop; it suppresses T_H2 cytokine production; it upregulates IL-12Rβ2, the receptor for the APC-derived T_H1-inducing cytokine IL-12 (43-45). The importance of T-bet in T_H1 differentiation is demonstrated in T-bet-deficient mice, which do not develop T_H1 cells and are susceptible to T_H1 cell-mediated infection (46). Regarding T_H2 cells, GATA-3 is necessary for the initiation of T_H2 differentiation and is indispensable for the production of the T_H2 cytokines IL-5 and IL-13 (47-49).

Beyond T_H1 and T_H2 subsets

In addition to the canonical T_H1 and T_H2 cell subtypes, additional T_H subsets exist that are characterised by different effector functions. These include TGFβ-secreting T_H3 cells, IL-10-

secreting Tr1 cells, natural T regulatory (Tregs) cells and IL-17-producing T_H17 cells. T_H3, Tr1 and Treg cells are all regulatory $CD4^+$ T cells that can suppress adaptive T cell responses and thus have an important role in controlling autoimmunity (50). While T_H3 and Tr1 cells develop from $CD4^+$ T cells in the periphery, Tregs represent a diverse lineage that develops intrathymically. T_H17 cells, on the other hand, are not regulatory and form a new branch of $CD4^+$ effector T cell distinct from T_H1 and T_H2 cells (51;52). Differentiation of naïve $CD4^+$ T cells into polarised T_H17 cells *in vitro* occurs through stimulation by APC-derived cytokines TGFβ and IL-6, a process that is amplified by IL-1β and is negatively regulated by T_H1 and T_H2 cytokines (53) (**Fig. II**). Meanwhile, survival and expansion of T_H17 cells is thought to rely on IL-23 stimulation. Both *in vitro* and *in vivo* differentiation of T_H17 cells require induction of the transcription factor retinoic acid-related orphan receptor-γt (RORγt), which is characteristic of this new T cell subset (54). T_H17 cells are characterised by the secretion of the cytokine IL-17 (also known as IL-17A) but they also produce IL-17F (another IL-17 family member with closest sequence identity to IL-17A) , IL-6, tumour necrosis factor (TNF) (55;56) and IL-22 (57-59). IL-17 is a proinflammatory cytokine that induces the expression of IL-1, IL-6 and granulocyte colony stimulating factor (G-CSF), as well as chemokines, by cells such as fibroblasts, stromal cells and endothelial cells (60-66). Therefore, in contrast to T_H1 and T_H2 cells, which aid the development of a specific immune response and have effects on non-immune target cells, the effects of IL-17 so far appear to be limited to non-immune cells.

APC-DERIVED CYTOKINES IN T CELL DIFFERENTIATION

APC-derived cytokines constitute signal 3 of T cell activation and critically, they determine the outcome of T cell differentiation giving them a significant role in governing immunity. Until now, no APC-derived factor has been shown to induce T_H2 development however IL-12 and IL-18 are known to be major commanders of T_H1 polarisation (**Fig. II**).

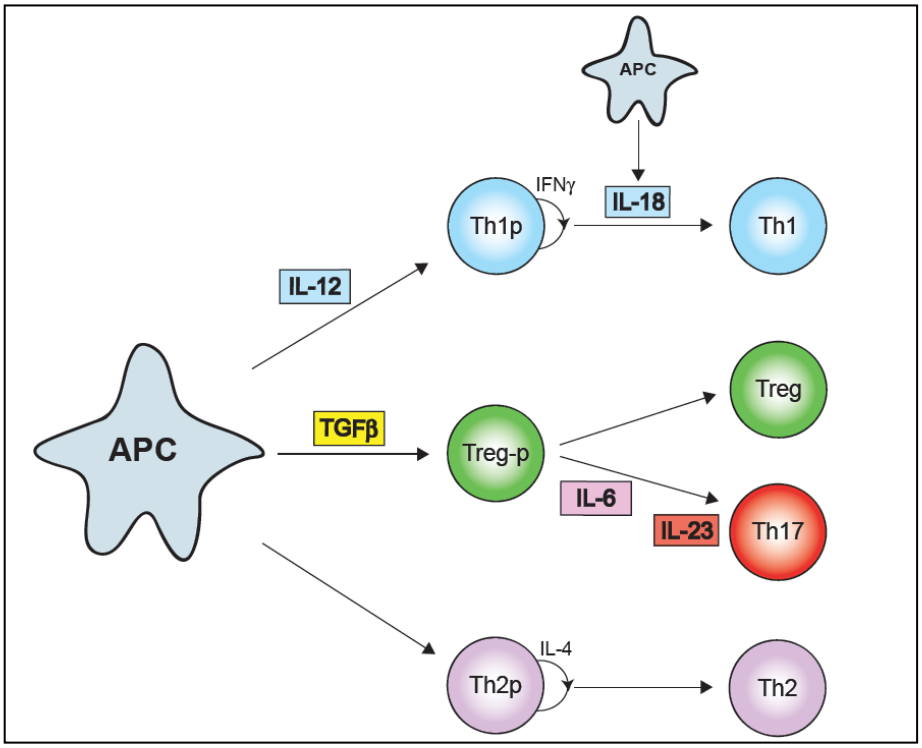


Figure II: APC-derived cytokines guide the differentiation of naïve T cells to an effector T cell subtype. Secretion of IL-12, in synergy with IL-18, leads to the generation of Th1 cells. Initial IL-12 production directs the upregulation of IL-18R and IL-12Rβ2 expression on the surface of T_H1 precursor (T_H1p) cells that allows IL-18 to aid IL-12 in T_H1 polarisation. TGFβ secretion can polarise naïve cells towards a regulatory phenotype or an auto-aggressive phenotype depending on the cytokine environment: secretion of TGFβ alone by APCs supports regulatory T cell (Treg) formation, from Treg precursor (Treg-p) cells, which counteracts autoimmune inflammation. However, the additional presence of IL-6 results in the production of T_H17 cells, which are now considered the pathogenic T cell population during autoimmunity. The pathogenic APC-derived cytokine IL-23 is critical for the maintenance and survival of these auto-reactive T_H17 cells. The interaction of APCs and T_H2p cells and the absence of IL-12 and IL-18 induce the production of the T_H2 cytokine IL-4 from T cells, which acts in an autocrine fashion to polarise committed T_H2 cells.

IL-12 – the Major T_H1-Inducing Cytokine

IL-12 is a heterodimeric cytokine of 70 kDa consisting of heavy (p40) and light (p35) subunits, which are covalently linked (67-69). IL-12 is produced mainly by APCs, in particular by DCs and macrophages, although it can also be secreted by PMNs. While p40 appears to be produced in abundance in these cell types, the production of p35 is ubiquitously and constitutively expressed only at low levels and it requires p40 coexpression for secretion of the biologically active cytokine from the cell (70). IL-12 is mostly secreted upon TLR activation and it therefore acts as an early proinflammatory cytokine in response to many infectious agents (24). IL-12 signalling has a multitude of effects during an immune response: It acts upon NK cells and $CD8^+$ T cells to increase their cytotoxic activities; it stimulates the production of cytokines, in particular and most importantly IFNγ but also GM-CSF, TNF and IL-2 that are necessary for the ensuing immune response; it drives further production of IL-12 from DCs and macrophages in a positive feedback loop and finally it drives the differentiation of naïve T cells to an IFNγ-producing T_H1 effector cell phenotype (71). Thus, IFNγ is an important mediator of the effects of IL-12. IL-12 is the most critical cytokine promoting T_H1 differentiation and expansion although its actions can be influenced by the presence of other cytokines. IL-18, for example, synergises with IL-12 in T_H1 differentiation (**Fig. II**) while T_H2 cytokines such as IL-4, IL-5 and IL-13 reciprocally oppose IL-12 and T_H1 development (71).

IL-12 signals through the heterodimeric IL-12 receptor (IL-12R) which is formed by IL-12Rβ1 and IL-12Rβ2 subunits and which predominantly bind to p40 and p35 chains, respectively (72). Coexpression of both subunits is required for the generation of high affinity binding sites for p70 binding and IL-12Rβ2 is the critical signal transducing subunit of the receptor complex (73). The IL-12R is predominantly found on NK cells and activated T cells but it has also been demonstrated on the surface of APCs implying an autocrine effect

(74;75). The expression of IL-12Rβ2 on T_H1 cells is tightly regulated with its expression being maintained by IFN-γ and T-bet expression and inhibited by the T_H2 cytokine IL-4.

IL-18 – a Pleiotropic Cytokine

IL-18 is an 18 kDa proinflammatory cytokine and a member of the IL-1 superfamily of cytokines although its activities are more similarly related to those of IL-12. In fact, this cytokine was initially described as the IFNγ-inducing factor (IGIF) (76). IL-18 is expressed by macrophages and DCs in response to TLR activation but is also produced by epithelial cells, keratinocytes and other stromal cells (77). It is produced as a 24 kDa inactive precursor protein (pro-IL-18) lacking a signal peptide and, like IL-1β, must be cleaved by caspase-1 (also called IL-1β-converting enzyme (ICE)) to its biologically active form (76;78;79). IL-18 signals through the IL-18R, a heterodimeric complex of an IL-18Rα-binding subunit and an IL-18Rβ signalling subunit (also termed IL-1RAcPL or IL-1R7) (80-82). The extracellular portion of IL-18Rα is responsible for IL-18 binding. However this requires the presence of the receptor β-subunit, which composes the high affinity receptor complex. IL-18R is expressed by a variety of immune cells including lymphocytes, NK cells, macrophages and neutrophils (83-85). As a result, IL-18 has pleiotropic effects although it is best known for stimulating the cytotoxic activities of NK cells and promoting T_H1 differentiation and IFNγ production through its synergistic activities with IL-12 (**Fig. II**). The synergism of IL-12 and IL-18 in T_H1 polarisation results from the reciprocal enhancement of their signalling pathways (86-88) as well as the upregulated expression of each other's receptors on Th1 cells (35;36;89). Interestingly, despite its prominent feature as a T_H1 inducer, IL-18 can also promote T_H2 cytokine production in the absence of IL-12 and in an IL-4-dependent manner (77).

IL-18R activation initiates a downstream signalling pathway that is shared with other IL-1 and TLR family members (90). This includes recruitment of MyD88 to the receptor

complex and phosphorylation and activation of IL-1R-associated kinase (IRAK) and the adaptor molecule tumor necrosis factor receptor-associated factor 6 (TRAF6). Intracellular signalling ultimately leads to the phosphorylation and degradation of the inhibitory IκB kinases thereby liberating the transcription factor NFκB and permitting its translocation to the nucleus for gene transcription. In addition, IL-18R signalling has been demonstrated to activate members of the MAPK family.

THE ROLE OF CELL-MEDIATED IMMUNITY IN AUTOIMMUNE INFLAMMATION

T_H1/T_H2 Paradigm of Autoimmunity

Until recently, the development of autoimmune diseases was also governed conceptually by the T_H1/T_H2 subset model, whereby it was widely held that T_H1 cells form the pathogenic T cell subset in the context of tissue specific T cell driven autoimmune diseases, while T_H2 cells are thought to mediate allergic responses and asthma. Conversely T_H2 cells were reported to exert beneficial function during tissue-directed autoimmune disease pathogenesis (91). With regards to autoimmune inflammation of the CNS, this paradigm was proposed on the basis of several observations: tissue-invading T_H cells normally express IFNγ (92); Th1-inducing cytokines are present in the inflammatory lesion and often correlate with disease severity (93); EAE can be induced by the adoptive transfer of encephalitogenic T_H1 cells (94;95). Indeed treatment of mice with T_H1-inducing cytokines mostly results in aggravation of autoimmune diseases, such as EAE (for a list of reports in support of the T_H1/T_H2 paradigm of autoimmunity see **Table I**).

However, in stark contrast to this data, the generation and immunisation of mice deficient in T_H1 cytokines such as IFNγ and TNFα demonstrated that they were not, in contrast to expectations, protected from autoimmune disease. Disease progression and severity of both EAE in TNFα-deficient mice was identical to that in wt mice, while IFNγ-

and IFNγR-, deficient mice were hypersusceptible to disease (96;97). Loss of IFNγ even causes disease resistant mouse strains to develop EAE (98). In addition, deletion of T_H2 cytokines generally did not affect the progression of autoimmune disease (99). The disease-exacerbating effects of T_H1 cytokine deficiency thus presents a dichotomy as it suggests protective effects for T_H1 cytokines. Despite these contradictory results, autoimmunity has persisted to be defined by the T_H1/T_H2 paradigm mostly due to data describing autoimmune disease in mice deficient either in the transcription factor for IFNγ or in T_H1-inducing cytokines. Consistent with a role for T_H1 cells and IFNγ in autoimmunity, mice deficient in the T_H1 transcription factor T-bet are resistant to the induction of several experimental autoimmune diseases, such as EAE, inflammatory bowel disease (IBS) and SLE (100-103). Protection from disease correlated with a skewing in the ratio of the T_H1/T_H2 response towards a more dominant T_H2 environment that included significant upregulation of the T_H2 cytokines IL-4, IL-5 and IL-10. Conversely, inhibition of the T_H2 transcription factor GATA-3 reduces T_H2 cytokine expression thereby decreasing allergic airway inflammation and hyper-responsiveness (104). Such an imbalance in the T_H1/T_H2-inducing transcription factors has also been observed during human disease whereby relapsing-remitting MS patients have an increased expression of T-bet in peripheral blood leukocytes during relapse (105). On the other hand, increased gene expression of GATA-3 and reduced expression of T-bet have been demonstrated in the airways of patients with asthma (106;107). In light of the above data (**Table I**), immune modifying strategies for the treatment of autoimmune inflammation have focused on the concept of immune deviation (for review see Nicholson & Kuchroo) (108), whereby auto-aggressive T_H1 cells are deviated towards a T_H2 polarized phenotype. Taken together, while the $T_H1/2$ paradigm of autoimmunity has been bombarded with conflicting data, the fact that T-bet-deficient mice, as well as mice deficient in APC-derived T_H1 inducing cytokines, are resistant to the induction of Ag-driven autoimmune diseases has held

this paradigm firmly in place. I will now discuss the role of APC-derived cytokines and the paradigm shift that was finally initiated by a number of key findings.

Evidence for a Pathogenic Role of IL-12 in Autoimmunity

APC-derived cytokines constitute signal 3 of T cell activation and, critically, they determine the outcome of T cell differentiation giving them a significant role in governing autoimmunity. Mice deficient in the T_H1-inducing cytokines IL-12 and IL-18 have been shown to be resistant to EAE, again coinciding with reduced T_H1 development. In addition, the treatment of mice with neutralizing antibodies against IL-12p40 effectively prevents disease induction and suppresses EAE in adoptive transfer recipients (109). The treatment of mice with recombinant IL-12 is capable of exacerbating EAE and is again thought to result from enhanced activation of IFNγ-producing T cells (110). Of significant importance, roles for Th1-inducing cytokines can also be derived from the human disease. In MS, patients demonstrate increased levels of IL-12 in serum (111), cerebrospinal fluid (CSF) (112), MS lesions and peripheral blood mononuclear cells (PBMC) when compared to controls (113). It has also been shown that augmented IL-12 expression is representative of the degree of disability whereby IL-12p40 mRNA is elevated in MS patients during the development of active lesions and immediately prior to relapses during relapsing-remitting MS (114). Therefore, data from human autoimmune disease as well as the animal model both point towards a highly relevant role for T_H1 inducing conditions and IFNγ production in autoimmune pathogenesis (**Table I**).

Table I: Evidence for T_H1 Pathogenesis in Autoimmunity	**References**
<u>T_H1 cytokines in multiple sclerosis</u>	
• ↑ IL-12 demonstrated in plasma, CSF, PBMCs and MS plaques of MS patients	(111-117)
• Higher percentage of IL-12Rβ1 and IL-12Rβ2-expressing T cells in CSF of MS patients compared to blood	(118)
• ↑ IL-18 expression in serum, PBMCs and demyelinating lesions of MS patients	(119-121)
• ↑ serum levels of IFNγ in RR-MS patients prior to onset of relapse	(122)
• Significant positive correlation between IFNγ-producing PBMCs and MS disability	(123)
• ↑ IL-12, IL-2, IFNγ, TNFα, IL-1β and IL-6 in PBMCs of acute and relapsing MS patients	(117)
<u>T_H1 cytokines in experimental autoimmune encephalomyelitis</u>	
• Anti-IL-12p40 Ab suppresses EAE in adoptive transfer recipients	(124)
• IL-12p40-deficient mice are resistant to MBP-induced EAE	(125)
• Anti-IL-18 Abs significantly ↓ IFNγ production and disease development during MBP-induced EAE in rats	(126)
• IL-18-deficient mice are resistant to MOG_{35-55}-induced EAE due to decreased IFNγ	(84)
• CNS-infiltrating cells of EAE mice stained for IFNγ and IL-2	(93)
• CD4+ T cells infiltrating the CNS of acute EAE mice produced the Th1 cytokines IL-2 and IFN-gamma	(92)
• T-bet-deficient mice completely fail to generate Th1 cells and thus are resistant to MOG_{35-55}-induced EAE	(100)

Evidence against a Pathogenic Function for IL-12 in Autoimmunity

As explained above, IL-12 is a heterodimeric cytokine consisting of p35 and p40 subunits. The wealth of data pertaining to IL-12's role in autoimmunity originated from using mice deficient in the p40 subunit of IL-12 as well as administering antibodies which turned out to be specific against IL-12p40, both of which abolish expression and/or the function of IL-12. However, a series of reports have led to a revised concept of IL-12 function in autoimmune disease, starting in 1999 with further evidence that the p40 subunit is able to dimerise with

subunits other than p35. While it was previously known that p40 could homodimerise to form p80 with putative IL-12 antagonistic activities, Oppmann and colleagues identified an IL-6/IL-12-related subunit, p19, with which p40 heterodimerises to form the cytokine IL-23 (127). Upon ubiquitous transgenic expression of p19 in mice, Wiekowski et al. could show that p19 has similar biological properties to IL-6, G-CSF and IL-12, which resulted in the induction of multi-organ inflammation in these animals (128). In 2002, Becher et al demonstrated that immunisation of mice deficient in IL-12p35 were surprisingly not resistant to MOG-induced EAE but actually had increased clinical severity, a finding that could also be reproduced by others (129;130). Interestingly, the conflicting phenotypes were accompanied by a decrease in T_H1 immunity in both genotypes. In addition, mice lacking the p40-binding subunit of IL-12, IL-12Rβ1, were also EAE resistant, but the p35-binding subunit, IL-12Rβ2, was shown to be irrelevant (131). The discordant effects of IL-12p40 and IL-12p35 clearly suggest an indispensable role for p40 in experimental disease but not for IL-12. The incompatibility of the p40 and p35 phenotypes seemed likely to result from the promiscuity of the p40 subunit, whereby resistance of p40-deficient mice resulted from the lack of IL-23 and not IL-12. This was later confirmed in mice deficient in the IL-23 subunit p19, and therefore in IL-23 alone, which in resemblance to p40-deficient mice, were protected from both EAE and CIA (132;133) (**Table II**). In contrast to p40-deficient mice, p19 deficiency does not impact on the development of T_H1 cells and IFNγ production, which provides further and overwhelming evidence against a critical pathogenic role for T_H1 in autoimmunity.

IL-23 is secreted by APCs and signals via the IL-23R that is made up of the p40-binding IL-12Rβ1 subunit and the signalling IL-23R subunit, which together form the high affinity receptor complex for IL-23 (134). IL-23R is present on the surface of various cells of the immune system including activated/memory T cells, NK cells, DCs, monocytes and macrophages (135). In contrast to IL-12R, IL-23R is highly expressed on murine memory $CD4^+$ T cells, and is expressed at low levels on naïve T cells, permitting unique effects for IL-

23 on this cell type. As demonstrated in $p19^{-/-}$ mice, IL-23 does not affect T_H1 polarization as observed by an intact IFNγ response (56). Alternatively, IL-23 is one of the factors essential for the expansion and survival of the newly-defined T_H17 cell.

T_H17 cells in Autoimmune Inflammation

T_H17 cells and the expression of IL-17 have been associated with numerous human autoimmune diseases including RA (136;137) and MS (138;139). Furthermore, its inhibition or deletion in the corresponding animal models has provided a varying degree of protection (56;140-143). In light of this emerging T cell subset, some of the previously observed discrepancies in the T_H1/T_H2 paradigm of autoimmunity could be explained, especially with regards to the conflicting data obtained for IL-12p40- and IL-12p35-deficient mice. Cells from EAE resistant IL-$12p40^{-/-}$ mice demonstrate both defective T_H1 and T_H17 responses while cells from hyper-susceptible IL-$12p35^{-/-}$ mice, which still express IL-23, lack T_H1 development but have an elevated frequency of T_H17 cells (51;144). Consistently, EAE-resistant IL-$23p19^{-/-}$ mice show normal T_H1 responses but have a significantly reduced production of IL-17. Adoptive transfer experiments showed that encephalitogenic $CD4^+$ T cells that had been treated *in vitro* with Ag and IL-23, but not IL-12, could induce EAE in recipient mice (56). In accordance with a role for IL-23 in the expansion and survival of T_H17 cells, IL-23 has been demonstrated to be required during the effector phase of EAE (132;145). The generation of bone marrow chimeric mice, in which p40 was expressed either in the CNS or the peripheral immune system, showed that p40 is not absolutely required for the infiltration of inflammatory cells into the CNS but that p40 produced by cells resident to the CNS is essential for maintaining encephalitogenicity of T cell migration into the CNS (145). Furthermore, Cua et al showed that injection of IL-23 into the CNS of $p19^{-/-}$ mice abrogated resistance of these animals to EAE (132). They suggest that the role of IL-23 in promoting encepalitogenicity results from its activation of macrophages, present in the inflamed CNS, to

produce proinflammatory cytokines such as IL-1 and TNF. This points to an important role for IL-23 in the regulation of myeloid cells in organ-specific autoimmune diseases. Therefore IL-23 and not IL-12 is required for mediating the pathogenesis of CNS autoimmune inflammation, which is subsequently, to some extent, modulated by the IL-12-IFNγ pathway (for a list of reports rejecting the T_H1/T_H2 paradigm see **Table II**).

Table II: Evidence against T_H1 Pathogenesis in Autoimmunity	**References**
• IL-12p35$^{-/-}$ mice are susceptible to EAE	(129;130;146)
• IL-23$^{-/-}$ mice are resistant to EAE	(132)
• IFNγ$^{-/-}$ mice are hypersusceptible to EAE	(96;98)
• IFNγ$^{-/-}$ and IFNγ receptor deficient mice are not protected from CIA	(147-149)
• TNFα$^{-/-}$ mice are not resistant to EAE	(97)
• IL-4$^{-/-}$ mice are not hypersusceptible to EAE	(99)
• IL-23 induces a population of encephalitogenic CD4$^+$ T cells called T_H17 cells	(56)
• IL-17 block or deficiency reduces susceptibility to EAE and CIA	(56;140;141;143)
• IL-17 mRNA is augmented in blood and CSF mononuclear cells of MS patients	(138)
• IL-17 is produced by the rheumatoid synovium	(136)

IL-18 in Autoimmune Inflammation

The above evidence clearly argues against a role for IFNγ-secreting T_H1 cells in the pathogenesis of autoimmune disease and suggests a more regulatory role for this subset. However the known actions of IL-18, as well as the previously discussed role of T-bet, have prevented completely dismissing this paradigm. Mice deficient in the T_H1-inducing cytokine IL-18 have been shown to be protected from MOG_{35-55}-induced EAE and are deficient in mounting auto-reactive T_H1 responses (84). The defective T_H1 cell response was suggested to

result at least in part from abrogated IFNγ production by NK cells, as transfer of NK cells from recombination activating gene (RAG)-deficient, but not from IFNγ$^{-/-}$, mice were able to rescue the susceptibility of these mice to EAE. In addition, the administration of anti-IL-18 antibodies (Ab) to MBP-immunised rats significantly reduced the production of IFNγ as well as disease development. With regards to human autoimmune disease, increased IL-18 expression has been demonstrated in the serum (119), PBMCs (121) and actively demyelinating lesions (120) of MS patients. These data point towards a role for IL-18 in CNS autoimmune inflammation at the level of T cell priming and T_H1 induction (**Table I**), which is surprising given the clearly protective effects of IL-12 and other T_H1 cytokines. This could suggest, however, that IL-18 is capable of compensating for the lack of IL-12 in IL-12p35$^{-/-}$ mice thereby implying redundancy in auto-reactive T_H1 cell development.

The role of IL-18 in CNS autoimmune inflammation has mainly focused on its influence upon T_H1 polarization and NK cell activation. With regards to the effector stage of disease, there is a lack of data pertaining to the role of IL-18 in MS and EAE pathology. IL-18 has been detected in the mouse brain *in vivo* and microglia were postulated to be the potential producers as cultured microglia was capable of producing IL-18 upon stimulation with LPS (150). Indeed, IL-18 mRNA expression has been shown to increase in the CNS during acute EAE and IL-18 induces the expression and release of cytokines from murine glial cells (151). In addition, IL-18 and IL-12 have been shown to induce IFNγ production and MHC II mRNA expression in microglia (152). Although Shi et al have demonstrated that IL-18$^{-/-}$ mice are susceptible to the adoptive transfer of encephalitogenic wt lymphocytes implying that IL-18 has no role in the CNS, it has been suggested that IL-18 may act to terminate the immune response in the CNS due to its role in Fas/Fas ligand mediated apoptosis (151). Taken together, the role of the T_H1-inducing cytokine IL-18 remains improperly characterised with regards to its actions in the CNS during the effector phase of EAE and in the context of the changing T_H1/T_H2 paradigm of autoimmunity.

SPECIFIC AIMS

The aims of this PhD thesis were:

1. The assessment of the role of IL-18 as a compensatory factor in IL-12-deficient mice during CNS autoimmune inflammation

2. Characterisation of the role of IL-18 in the effector stage of CNS autoimmune inflammation

3. Characterisation of the role of IL-18Rα in CNS autoimmune inflammation

MATERIALS AND METHODS

MICE

Female C57BL/6 mice were purchased from Harlan Laboratories (Netherlands). IL-12p35$^{-/-}$, IL-12p40$^{-/-}$ and OT-II (C57BL/6-Tg(TCRaTCRb)425Cbn/J) mice were purchased from Jackson Laboratories (USA). Homozygous IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ (all backcrossed onto C57BL/6 for more than 12 generations) were provided by S. Akira (Osaka) and Rag1$^{-/-}$ mice were provided by R. Zinkernagel (Zurich) and were bred in house under specific pathogen free (spf) conditions. The 2d2 (MOG-TCR-Tg) mice were provided by V. Kuchroo (Harvard Medical School, Boston, Massachusetts). Animal experiments and breeding were approved by the Swiss Veterinary Office (#69/2003 and #70/2003).

Bone Marrow Chimera Generation

Irradiation Bone Marrow (BM)-chimeric mice were generated as previously described (11). Briefly, BM-donor mice were euthanized using CO_2 and BM-cells were isolated by flushing femur, tibia, radius and hip bones with phosphate buffered solution (PBS). BM cells were then passed through a 100 μm cell strainer and cells were washed with PBS. Recipient mice were lethally irradiated with 1100 rads (split dose) and i.v. injected with 12-25x10^6 BM-cells. Engraftment took place over 8 weeks of recovery.

EAE INDUCTION AND EVALUATION

Active Immunisation

Mice were immunised subcutaneously with 200 μg of MOG_{35-55} (amino acid sequence: MEVGWYRSPFSRVVHLYRNGK), obtained from GenScript (Piscataway, NJ), emulsified in CFA (DIFCO, Detroit, MI). Mice received 200 ng pertussis toxin (PtX) (Sigma-Aldrich) intraperitoneally at the time of immunization and 48 hours later. Monoclonal anti-IL-18Rα antibody (clone 112624) (R&D Systems) was administered either 1 day pre-immunisation

(450 μg/mouse) and every 3 days thereafter (300 μg/mouse) or every 3 days beginning from disease onset (300 μg/mouse). Clinical disease usually commences between days 10 and 14 post-immunisation and between days 6 and 10 for BM-chimeric mice.

Passive Immunisation

For adoptive transfer, MOG-reactive T cells were generated as described (153). Briefly, donor mice were immunised with 200 μg of MOG_{35-55} peptide in CFA and given 200 ng PtX at the time of immunisation only. After 11 days, LNs and spleens were isolated, homogenised and the single cell suspensions were filtered through a 100 μm cell strainer before centrifugation at 1500 r.p.m. for 10 min at 4°C. For lysis of red blood cells (RBCs), splenocytes were incubated on ice for 10 mins in lysing buffer (containing NH_4Cl, $KHCO_3$ and EDTA). The cells were then washed with PBS, centrifuged and filtered a second time before counting. LN cells and splenocytes were resuspended and plated at a concentration of $4x10^6$ cells/ml in RPMI medium containing 10% FCS, 1% penicillin/streptomycin, 1% glutamine and 50 μM β-mercaptoethanol (RPMI complete) with the addition of 50 μg/ml MOG_{35-55} and 2.5 ng/ml of recombinant IL-12 (Peprotech). Cells were left in culture for 4 days at which point they were scraped from the culture plate, washed and counted. Mice were injected i.p. with $25x10^6$ cells and received 200 ng PtX several hours later and on day 2 post-injection. Clinical disease usually begins 6-10 days after cell transfer.

Clinical Evaluation

Mice were scored daily as follows: 0) no detectable signs of EAE; 0.5) distal tail limp; 1) complete tail limp; 2) unilateral partial hind limb paralysis; 2.5) bilateral partial limb paralysis; 3) complete bilateral hind limb paralysis; 3.5) complete hind limb paralysis and unilateral forelimb paralysis; 4) total paralysis of fore and hind limbs (score > 4 to be

euthanized); 5) death. Each time point shown is the average disease score of each group. Statistical significance was assessed using an unpaired Student's *t*-Test (∗ < 0.05; # < 0.01).

Histology

Mice were euthanised with CO_2, followed by transcardial perfusion with PBS and subsequent perfusion with 4% (weight/volume) paraformaldehyde (PFA) in PBS. The spinal column was removed and fixed in 4% PFA in PBS. The spinal cord was then dissected and paraffin-embedded prior to staining with either haematoxylin & eosin (H&E) or CD3, B220 and MAC-3 antibodies (BD Pharmingen) to assess infiltration of inflammatory cells. Luxol fast blue (LFB) stain was used to determine the degree of demyelination or amyloid precursor protein (APP) was used to assess the extent of axonal damage.

CELL ANALYSIS

Flow Cytometry

For cytofluorometric analysis we used the following antibodies diluted in facs buffer (PBS supplemented with FCS and sodium azide): anti-CD45 (30/F11), anti-CD4 (RM4-5), anti-Vα3.2 (RR3-16), anti-CD11b (M1/70), anti-GR-1 (RB6-8C5) and anti-B220 (RA3-6B2), anti-CD80 (16-101), anti-CD86 (GL1), anti-CD40 (3/23), anti-CD62L (Mel-14), anti-CD162 (2Ph1), anti-VLA-4 (R1-2), anti-CD5 (53-7.3), anti-CD25 (PC61), anti-Ly5.1 (104), anti-Ly5.2 (A20), anti-IL-18Rα (clone 112624), anti-IL-17 (TC11-18H10) and anti-IFN-γ (XMG1.2). All antibodies were purchased from BD Pharmingen except from anti-IL-18Rα (clone 112624), which was bought from R&D Systems.

For analysis of CNS invading cells, mice were euthanised with CO_2 and perfused intracardially with PBS. Spinal cord was flushed out with PBS and the brain was dissected to isolate the brainstem. Tissues were digested using liberase treatment (400 μg/ml liberase (Roche) and 200 μg/ml DNase (Roche)) at 37°C for 30 min. The tissues were then

homogenised and strained through a 100μm nylon filter (Fisher). After centrifugation, cell suspensions were resuspended in 30% Percoll (Pharmacia) and centrifuged at 18,500g for 30 min at 4°C. Interphase cells were collected and washed extensively before being staining. For flow cytometry, we incubated the primary antibody for 20 min at 4°C. Cells were then washed with facs buffer and centrifuged for 2 min at room temperature (RT) prior to staining for 15 min with the secondary antibody at 4°C. Cells were washed, centrifuged and resuspended in 300 μl facs buffer for subsequent analysis. We analysed the cells using a FACSCalibur (BD Pharmingen) with CellQuest software. Post-acquisition analysis was performed using WinMDI 2.8 software (Scripps-Research Institute).

Recall Assays

Spleen, axillary and inguinal lymph nodes were isolated from naïve mice or mice primed by injections of 100 μg/flank of MOG_{35-55} or KLH (Sigma) emulsified in CFA 7 days earlier. $2x10^5$ cells were placed as triplicates in 96-well plates and stimulated for 48 hours with either 50 μg/ml KLH, 5 μg/ml ConA or medium. After 24 hours, 0.5 μCi/ml ^{3}[H]-thymidine was added to the wells and incubated for an additional 24 hours to observe proliferative responses. Thymidine incorporation was assessed using a Filtermate Harvester and a scintillation and luminescence counter. Briefly, samples were harvested onto glass fibre filter paper (Wallac) using a Filtermate Harvester. The filter paper was microwaved for 4 min to dry and placed in a plastic sample bag (Perkin Elmer), which was subsequently filled with 4.5 ml scintillation fluid. Scintillation fluid was spread over the filter and the remainder was expelled from the bag. The bag was then sealed and placed in the luminescence counter for detection of beta counts. For cytokine analysis, the culture supernatant of identical sister cultures was harvested after 48 hours and analysed in duplicate for IFN-γ, IL-17 and IL-23p40 production by ELISA (Pharmingen, La Jolla, CA).

ELISpot

For ELISpot assays, lymphocytes were isolated from the CNS of MOG_{35-55}-immunised mice on days 9 and 14 dpi by differential Percoll centrifugation as described above. Cells (2×10^5) were plated in complete RPMI containing 50 μg/ml MOG_{35-55} in 96-well plates (Millipore) coated with 7.5 μg/ml anti-IFN-γ (AN18; Mabtech) or 2 μg/ml anti-IL-17 (TC11-18H10; BD Pharmingen) Abs. The plates were incubated at 37°C, 5% CO_2 for 18 (anti-IL-17) or 20 (anti-IFN-γ) hours at which point cells were discarded and plates were washed with PBS. Then, 0.5 μg/ml of biotin-conjugated anti-IFN-γ (R4-6A2; Mabtech) or 1 μg/ml of biotin-conjugated anti-IL-17 (TC11-8H4.1; BD Pharmingen) detection Abs was added and incubated at 25°C for 2h and 4 h, respectively. After plates were washed, streptavidin-alkaline phosphatase (Mabtech) was added, followed by incubation for 1 h at 25°C. Plates were washed with PBS and 75 μl of the substrate solution BCIP/NBT-plus (5-bromo-4-chloro-3-indolylphosphate-nitro blue tetrazolium) was added to the wells, which were developed until distinct spots emerged. Plates were analysed with an enzyme-linked immunospot reader (Immunospot; CTL).

CYTOKINE ANALYSIS

Naïve Cells

To detect cytokine secretion from naïve cells, LNs were isolated from naïve wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice, homogenised and $2x10^5$ cells were plated in triplicate in a 96-well plate. 5μg/ml ConA was used for stimulation for 16 hours and 5μg/ml anti-CD40 (FGK) was used for stimulation for 36 hours before analysing IFNγ or IL-23p40 production by ELISA (BD Pharmingen), respectively. $CD4^+$ T cells were purified from naïve splenocytes using BD IMag Magnetic Beads (BD Pharmingen) and stimulated with 5 μg/ml anti-CD3 (2c11) and 5 μg/ml anti-CD28 (37N) for 36 hours before analysing IFN-γ production by ELISA.

CNS Tissue

To detect cytokine expression in the CNS, naïve mice or mice with EAE were sacrificed with CO_2 and transcardially perfused with PBS. The spinal cord was flushed out and the brain was dissected to isolate the brainstem. The CNS was homogenised on ice for 15 min in a homogenisation buffer containing 20 mM Tris/HCl (pH 7.3), 140 mM NaCl, 0.5% Triton X-100, 2 mM activated sodium orthovanadate and 1 tablet/50 ml Complete protease inhibitor (Boehringer Ingelheim). After homogenisation, the suspension was sonicated 3 times for 10 sec on ice, ensuring that the sample didn't become too hot. The extracts were then cleared from myelin by centrifugation at 2000 rpm for 10 min at 4°C. The supernatant containing cytokines was then added directly to the ELISA plate to detect expression of IL-18 in the CNS (BD Pharmingen).

REAL-TIME PCR

RNA Isolation and cDNA Synthesis

Spleen, axillary and inguinal lymph nodes were isolated from naïve mice or mice primed by injections of 100 μg/flank of MOG_{35-55} or KLH emulsified in CFA 7 days earlier. $4x10^6$ cells were plate in 6 well plates and stimulated with 50μg/ml of the cognate Ag or medium. After 2 days, RNA was isolated from restimulated cells by TRIzol extraction (Invitrogen). For RNA isolation, 200 μl isopropanol was added per ml of TRIzol and then samples were shaken vigorously, incubated for 3 min at RT and then centrifuged at 8000 rpm for 15 min at 4°C. The colourless upper phase was collected, added to a new, sterile eppendorf tube and RNA was precipitated by mixing with 500 μl chloroform. After 10 min incubation at RT, the samples were centrifuged again at 8000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed once with 75% ethanol. Samples were centrifuged at 5000 rpm for 10 min at 4°C, the ethanol was discarded and the pellets were left to dry at RT prior to resuspension in RNase-free water. For cDNA synthesis, 1-5 μg RNA was reverse transcribed

using Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT) (Invitrogen), which uses single stranded RNA to synthesise a complementary cDNA strand in the presence of a primer. The following components (all from Invitrogen) are mixed gently together in a tube and heated at 37°C for 5 min prior to adding M-MLV RT: 5X First strand buffer, dNTPs, DTT, random primers and Recombinant Ribonuclease Inhibitor RNaseOUT. The mix is then added to the RNA and incubated at RT for 10 min, 37°C for 50 min and 70°C for 15 min to inactivate the reaction.

Real-time PCR Analysis

Diluted cDNA was mixed with the following components. Real-time PCR was carried out to analyse RNA expression of cytokines and chemokines using the following primer sequences:

- IL-17 forward: ATCAGGACGCGCAAACATGA
- IL-17 reverse: TTGGACACGCTGAGCTTTGA

- IFNγ forward: GCATTCATGAGTATTGCCAAG
- IFNγ reverse: GGTGGACCACTCGGATGA

- IL-23p19 forward: CTG TTG CCC TGG GTC ACT CA
- IL-23p19 reverse: CAC ACT GGA TAC GGG GCA CAT TA

- IL-23p40 forward: TTTGCTGGTGTCTCCACTCA
- IL-23p40 reverse: CATCTTCTTCAGGCGTGTCA

- IL-1 forward: CAACCAACAAGTGATATTCTCCATG
- IL-1 reverse: GATCCACACTCTCCAGCTGCA

- TNFα forward: CATCTTCTCAAAATTCGAGTGACAA
- TNFα reverse: TGGGAGTAGACAAGGTACAACCC

- Rantes forward: TGCCCACGTCAAGGAGTATTT
- Rantes reverse: TCTCTGGGTTGGCACACACTT

- IP-10 forward: CTGCCGTCATTTTCTGCCTC
- IP-10 reverse: CACTGGCCCGTCATCGATAT

- MCP-1 forward: CAGCAGCAGGTGTCCCAAA
- MCP-1 reverse: TGTCTGGACCCATTCCTTCTTG

- MIP1α forward: TTTTGAAACCAGCAGCCTTTG
- MIP1α reverse: TTGGAGTCAGCGCAGATCTG

GENERATION OF BM-DERIVED DCs

BM-derived DCs were generated as described (154). Briefly, BM-donor mice were euthanised using CO_2 and the femurs and tibia were removed. BM-cells were isolated by flushing the bones with PBS and were filtered through a 100μm cell strainer. Cells (2-2.5x10^6 in 10 ml) were cultured in RMPI containing 10% FCS with the addition of 10% conditioned RPMI medium obtained from the culture of GM-CSF-transfected X-63 cells (obtained from A. Rollink, University of Basel, Switzerland). After at least 6 days, BM-derived DCs were matured with 10 μg/ml LPS overnight while immature BM-derived DCs were maintained in GM-CSF-containing medium. BM-derived DCs were used from days 7 to 9.

T CELL PROLIFERATION

Tg T Cell Proliferation and Polarisation

For *in vitro* proliferation of transgenic T cells, spleens were harvested from naïve OT-II mice and $CD4^+$ T cells were purified using BD-IMag magnetic beads (BD Pharmingen). The purity of T cell isolation was verified by FACS analysis. 1x10^5 OT-II T cells were cultured in a 96-well plate together with 3,000-10,000 immature or mature BM-DCs. Prior to co-culture, BM-DCs were pulsed with 1 μg/ml OVA protein (Sigma) in RPMI for 3 hours, followed by washing and irradiation with 2000 rads. Non-pulsed DCs were used as a control as well as T cells cultured alone. Cells were incubated for 4 days and ^{3}[H]-thymidine was added for the last 18 hours of culture.

For *in vivo* polarisation of TCR Tg cells, mice were injected with 25x10^6 OT-II splenocytes and immunized by bilateral flank injection of 200μg OVA protein emulsified in CFA. After 5

days, mice were killed and LN cells were isolated and incubated for 5 hours with 50 ng/ml phorbol 12-myristate 13-acetate (PMA), 500 ng/ml ionomycin and Golgiplug (BD Pharmingen). Cells were permeabilised with the Cytofix/Cytoperm Kit (BD Pharmingen) according to the manufacturer's protocol and stained with anti-IL-17, anti-IFNγ antibodies and an antibody against the OT-II Tg TCR, anti-Vα2, prior to cytofluorometric analysis.

Mixed Lymphocyte Reaction

To assess proliferation of allogeneic $CD4^+$ T cells, spleen and LNs were harvested from Balb/c mice and $CD4^+$ T cells were purified using BD-IMag magnetic beads (BD Pharmingen). $1x10^5$ $CD4^+$ T cells in complete RPMI were added to a 96-well plate. Mature and immature BM-DCs were washed, resuspended in RPMI at various concentrations and added to the $CD4^+$ T cells in triplicate. The MLR assay was incubated for 4 days at 37°C and ^{3}[H]-thymidine was added for the last 18 hours of culture.

MIGRATION ASSAYS

Migration assays were performed to analyse the migration capacities of neutrophils of DCs isolated from wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice. For isolation of neutrophils, mice were injected i.p. with 1 ml of 3% (w/v) Brewer's thioglycollate medium (Difco). To remove peritoneal exudates cells, the peritoneal cavity was flushed with 10 ml HBSS (without Ca^{2+} and Mg^{2+}) containing 1% BSA and 10 mM EDTA 20 hours post-injection. Cells were collected by centrifugation and Fc block (anti-CD16; BD Pharmingen) was added to the cells prior to FACS staining with anti-GR1 and anti-CD11b antibodies. Stained cells were filtered through a 50 μm mesh and neutrophils were FACS sorted for by gating on the double-positive GR1, CD11b population.

Sorted neutrophils were resuspended at $1x10^6$ cells/ml in X-vivo 15 medium (Biowhittaker/Cambrex) and 100 µl of the cell suspension was seeded into a transwell insert (Costar). The insert was added to a 24-well plate containing medium or the chemoattractant fMLP (end conc 10^{-6} M; Sigma). Cells were incubated for 60 min at 37°C and after incubation the remaining cells on the upper membrane were removed with a cotton swab. The migrated cells, attached on the lower part of the membrane, were stained for 20 min at 37°C with 1µM DAPI (Molecular Probes) in PBS and then fixed in 4% PFA. Migrated cells were counted with a square graticule (Leica Microsystems) in five visual fields per filter.

RESULTS

Mice deficient in both IL-12p35 and IL-18 are fully susceptible to EAE

EAE is generally referred to as a T_H1-mediated disease yet deletion of IL-12p35, a major T_H1-promoting cytokine, renders mice susceptible to MOG-peptide-induced EAE (129). Shi et al. have demonstrated, however, that mice deficient in IL-18, a cytokine that synergises with IL-12 in T_H1 polarisation, are resistant to EAE (84). To assess whether IL-18 is capable of compensating for the loss of IL-12 in $p35^{-/-}$ mice, rendering them EAE susceptibility, we generated mice deficient in both IL-12p35 and IL-18 ($p35^{-/-}$x $IL\text{-}18^{-/-}$). Immunisation with $MOG_{35\text{-}55}$ emulsified in CFA showed that $p35^{-/-}$x $IL\text{-}18^{-/-}$ mice are fully susceptible to EAE and have a similar disease severity and development as is seen in wt mice (**Fig. 1a and Table 1**). This demonstrates that IL-18 is not responsible for the EAE susceptibility of $IL\text{-}12p35^{-/-}$ mice and implies that IL-18 itself is a cytokine that has little or no effect on EAE pathogenesis. To confirm this, we actively immunised wt and single-mutant $IL\text{-}18^{-/-}$ mice with MOG peptide and found that $IL\text{-}18^{-/-}$ mice are indeed fully susceptible to EAE (**Fig. 1b and Table 1)**.

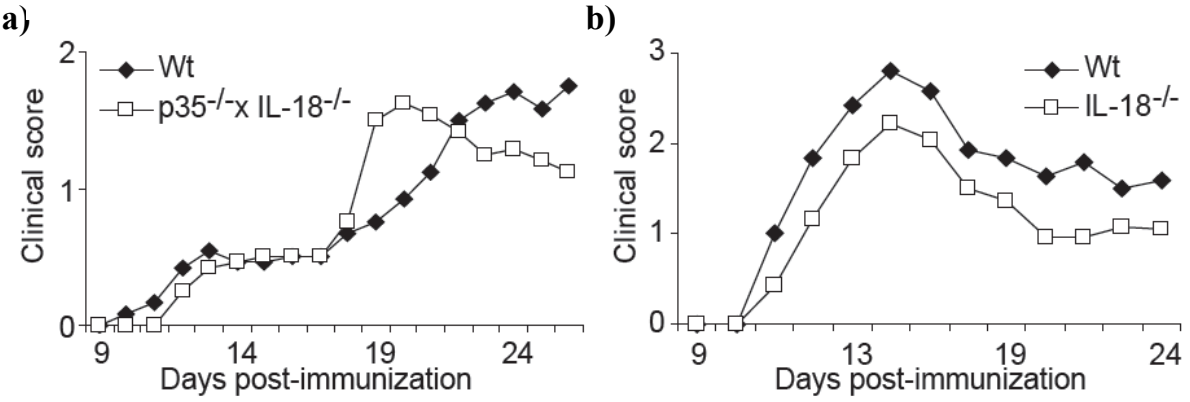


Figure 1: IL-18 is not required for EAE induction. EAE was induced by subcutaneous immunisation with $MOG_{35\text{-}55}$ emulsified in CFA. Data display the mean EAE score of each group. (**a**) EAE progression in $p35^{-/-}$x $IL\text{-}18^{-/-}$ double deficient and wt mice. Shown is one representative of 2 experiments ($n \geq 5$ mice/group). (**b**) EAE progression in wt and $IL\text{-}18^{-/-}$ mice. Shown is one representative of 3 individual experiments.

Table 1 EAE susceptibility of mice

Genotype	Incidence	Disease onset	Maximum clinical score
Wt	25 of 30 (83.33)	12.5 d	2.56 +/- 0.12
Il12p35$^{-/-}$*Il18*$^{-/-}$	7 of 10 (70)	14.1 d	3.20 +/- 0.08
Il18$^{-/-}$	20 of 22 (91)	12.8 d	2.35 +/- 0.13
Il18r1$^{-/-}$	2 of 20 (10)	18.5 d	2.60 +/- 0.12

EAE disease incidence (%), mean time of disease onset (d) and mean maximum clinical score (± S.E.M.) of diseased mice, for wild-type, IL-12p35$^{-/-}$IL-18$^{-/-}$, IL-18$^{-/-}$ and IL-18R$\alpha^{-/-}$ mice in which EAE was induced by subcutaneous immunisation with MOG_{35-55}.

IL-18 knockout mice are deficient in IL-18

Due to the fact that the development of EAE in IL-18$^{-/-}$ mice contradicted a previously published report (84), we extensively verified the targeting strategy and genotype of the mice. We could show that genomic DNA from IL-18$^{-/-}$ mice, in contrast to that of wt mice, is negative for the band amplifying the deleted sequence of IL-18 while being positive for the band amplifying part of IL-18 and the inserted neomycin cassette sequence (**Fig. 2a**). We also analysed by ELISA whether we could detect IL-18 secreted from activated splenocytes derived from wt and IL-18$^{-/-}$ mice, which showed that IL-18$^{-/-}$ mice are indeed completely IL-18 deficient (**Fig. 2b**). In addition, we could clearly establish that IL-18 is not present in the CNS of IL-18$^{-/-}$ mice, in contrast to wt mice (**Fig. 2c**). Therefore the susceptibility of IL-18-deficient mice to EAE, which is in contrast to the previously published data, did not occur due to the presence of a truncated yet functional form of IL-18 in these mice.

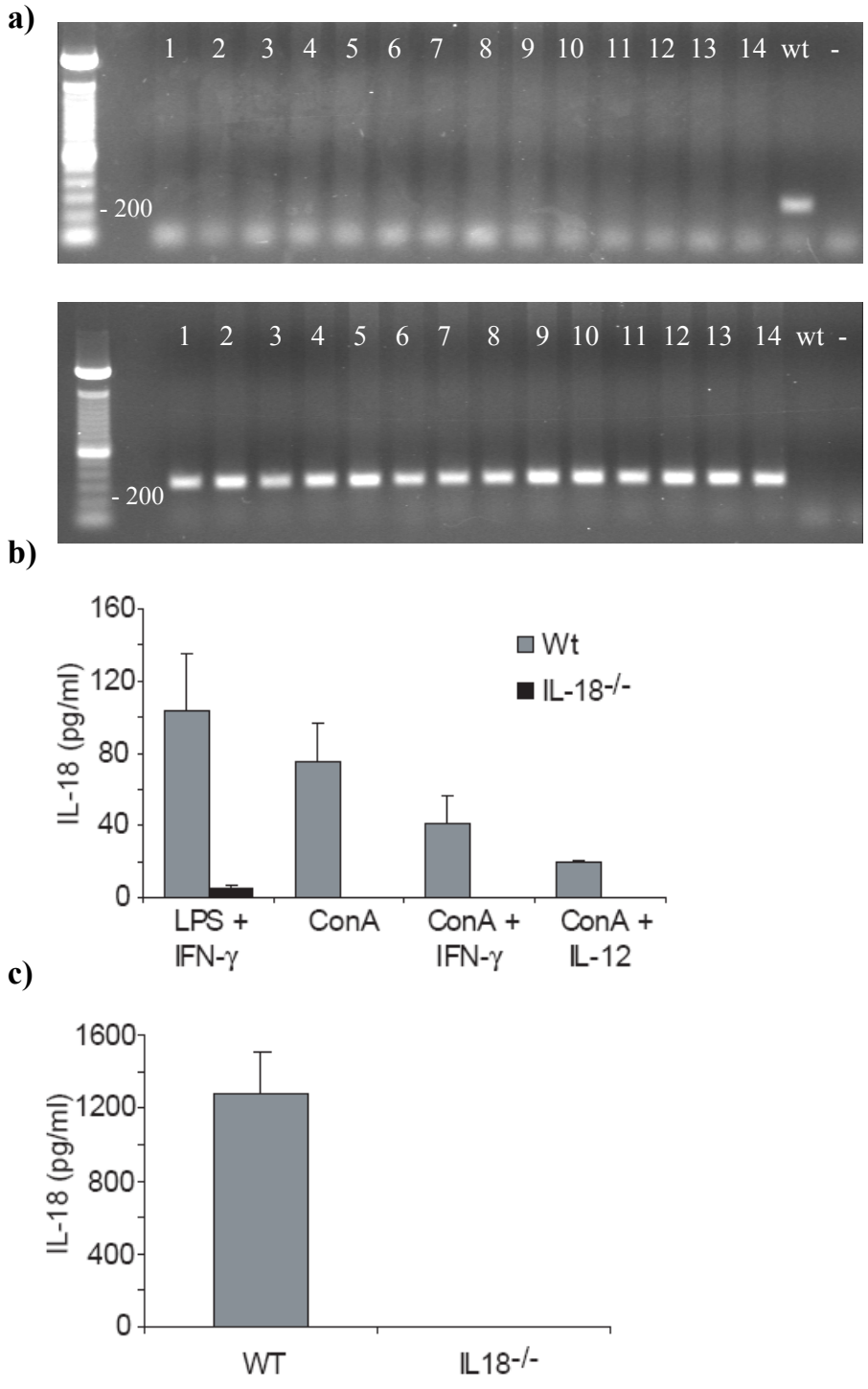


Figure 2: IL-18 is absent in IL-18-deficient mice. (**a**) IL-18-deficient tail DNA was amplified by PCR using primers for a sequence binding IL-18 (upper panel) or binding IL-18 and the neomycin cassette (lower panel). Wt DNA (wt) was used as a positive control and water (-) as a negative control. (**b**) ELISA of IL-18 production by wt and IL-18$^{-/-}$ splenocytes activated with LPS and IFNγ, ConA or a combination of ConA and IFNγ or IL-12. (**c**) ELISA measuring IL-18 expression in CNS (brain and spinal cord) of MOG_{35-55}-immunised wt and IL-18$^{-/-}$ mice 21 d.p.i.

IL-18 is required for Innate Immunity but not Ag-driven Immunity

Despite the fairly conclusive evidence for the lack of IL-18 in the IL-18-deficient mice, we decided to test this result further by using cells isolated from IL-18-deficient mice in an immunoassay. As observed in many experimental systems, the deletion of IL-18 consistently results in the paucity of an IFNγ response (155;156). We thus stimulated lymphocytes derived from naïve wt, IL-18$^{-/-}$ and, in addition, IL-18Rα$^{-/-}$ mice *in vitro* with the lectin Concanavalin A (ConA) for 16 hours and IFNγ production was subsequently measured by ELISA. Consistent with the principle that IL-18 has an effect on IFNγ production, lymphocytes from both IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice did not secrete IFNγ in contrast to wt lymphocytes (**Fig. 3a)**. We could further verify that T cell function and activation during polyclonal stimulation is not directly impaired as there was no difference in IFNγ secretion by stimulated wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ purified CD4^{+} T cells (**Fig. 3b**).

To establish the role of IL-18 and IL-18Rα in an adaptive Ag-driven immune response, we immunised wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice subcutaneously with MOG_{35-55} or with keyhole limpit hemocyanin (KLH), as a large immunogenic protein antigen (of about 400 kilodaltons) in CFA and 7 days later isolated and restimulated lymphocytes with the cognate Ag *in vitro*. Surprisingly, when KLH was used, we did not observe a drastic difference in the IFNγ-producing ability of lymphocytes derived from wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice (**Fig. 3d**). The less immunogenic MOG-peptide however revealed that IL-18Rα$^{-/-}$ mice produced significantly less IFNγ than wt or IL-18$^{-/-}$ mice (**Fig. 3c**). Our data support the notion that while IL-18 and IL-18Rα are critical co-factors for the early IFNγ response of mitogen-activated T cells, peptide-induced responses revealed discordant behaviour between lymphocytes lacking IL-18 or IL-18R. On the other hand, activation and IFNγ production through a complex large protein Ag appears to be independent of IL-18 or IL-18Rα, a concept that is in agreement with the recent report by Santos et al (157).

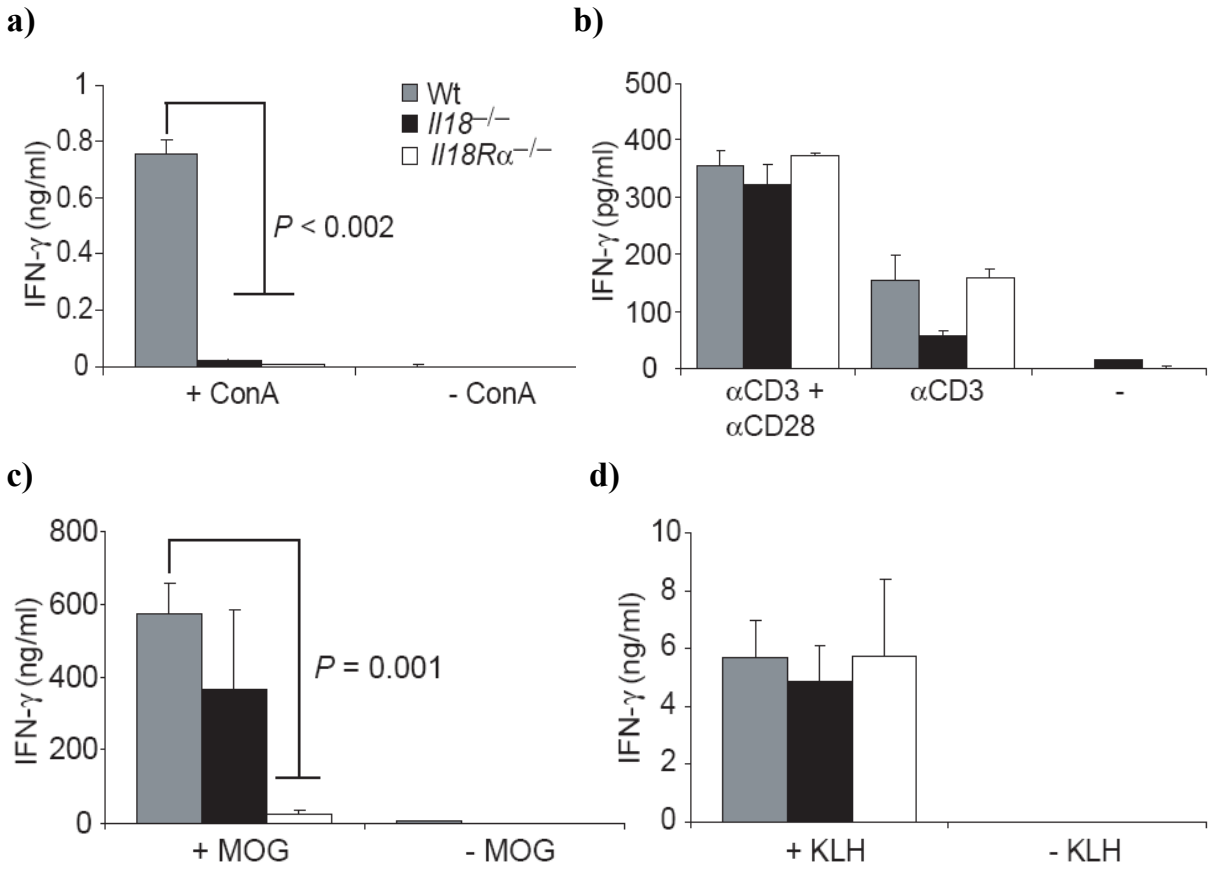


Figure 3: IL-18 is required for mitogen-, but not antigen-driven T_H1 development. (**a**) ELISA assessing IFNγ secretion by naïve wild-type, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ lymph node cells, stimulated for 16 hours with 5 μg/ml ConA (+ ConA) or medium (- ConA). (**b**) ELISA for IFNγ production from $CD4^+$ T cells purified from naïve wild-type, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ splenocytes by magnetic cell sorting and stimulated with anti-CD3 or a combination of anti-CD3 and anti-CD28 mAbs for 36 hours *in vitro* (**c,d**) Mice were immunised with 200 μg MOG_{35-55} or KLH in CFA and 7 days later LN cells were isolated and restimulated. (**c**) ELISA of IFNγ in supernatant from MOG_{35-55} immunised mice restimulated in duplicate with 50 μg/ml MOG_{35-55} (+ MOG) or medium (- MOG) for 48 hours. (**d**) ELISA of IFNγ in supernatant from KLH-immunised mice restimulated in duplicate with 50 μg/ml KLH (+ KLH) or medium (- KLH) for 48 hours. Error bars represent ± S.E.M. Data are representative of at least 2 individual experiments and combine 2 mice in each group.

IL-18Rα$^{-/-}$ mice are resistant to EAE

Mice deficient in IL-18Rα have been described as having an immunological phenotype similar to that of IL-18$^{-/-}$ mice (158). Given the discordant behaviour of IL-18- and IL-18Rα-deficient lymphocytes towards MOG-peptide in recall-responses, we discovered that IL-18Rα$^{-/-}$ mice were, in sharp contrast to both wt and IL-18$^{-/-}$ mice, resistant to EAE induction (**Fig. 4a** and **Table 1**). To ensure the absence of a truncated form of IL-18Rα in the IL-18Rα$^{-/-}$ mice, we tested for its presence in blood using an antibody specific for the extracellular, non-deleted part of the molecule. This showed that there was no IL-18Rα expression on cells from IL-18Rα-deficient mice (**Fig. 4b**). Histological analysis of spinal cords obtained 28 days after EAE induction showed that EAE-susceptible wt and IL-18$^{-/-}$ mice incur significant inflammation and demyelination as revealed by H&E and LFB staining, respectively (**Fig. 5a**). A more detailed analysis demonstrated the infiltration of T cells, macrophages and B cells and axonal damage in the CNS of these mice (**Fig. 5a** and **Fig. 5b**). In contrast, IL-18Rα$^{-/-}$ samples demonstrated no leukocyte infiltration or demyelination (**Fig. 5a** and **Fig. 5b**). Quantitative RNA analysis of spinal cords from mice with EAE showed that in accordance with the presence of inflammatory infiltrates, wt and IL-18$^{-/-}$ mice had increased expression of chemokines and inflammatory cytokines while these were decreased or absent in IL-18Rα$^{-/-}$ mice (**Fig. 6**).

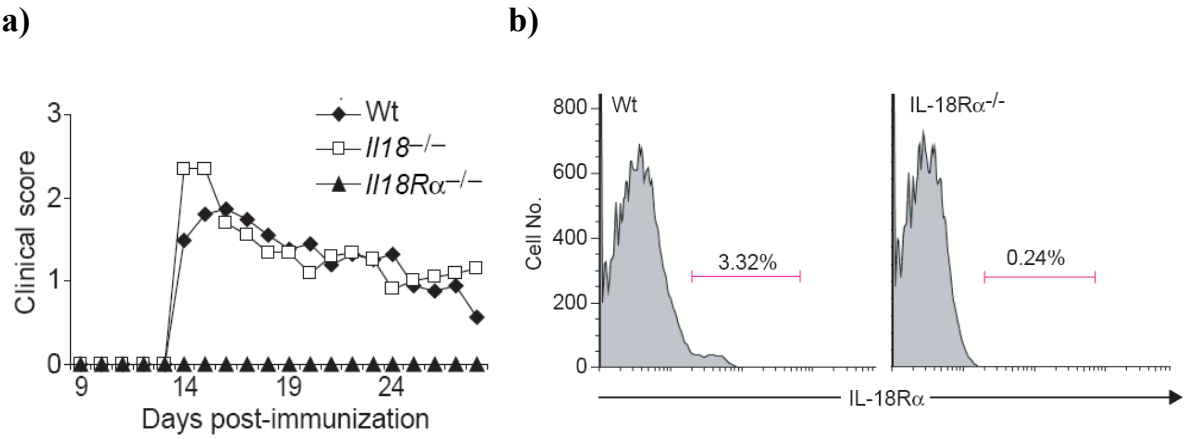
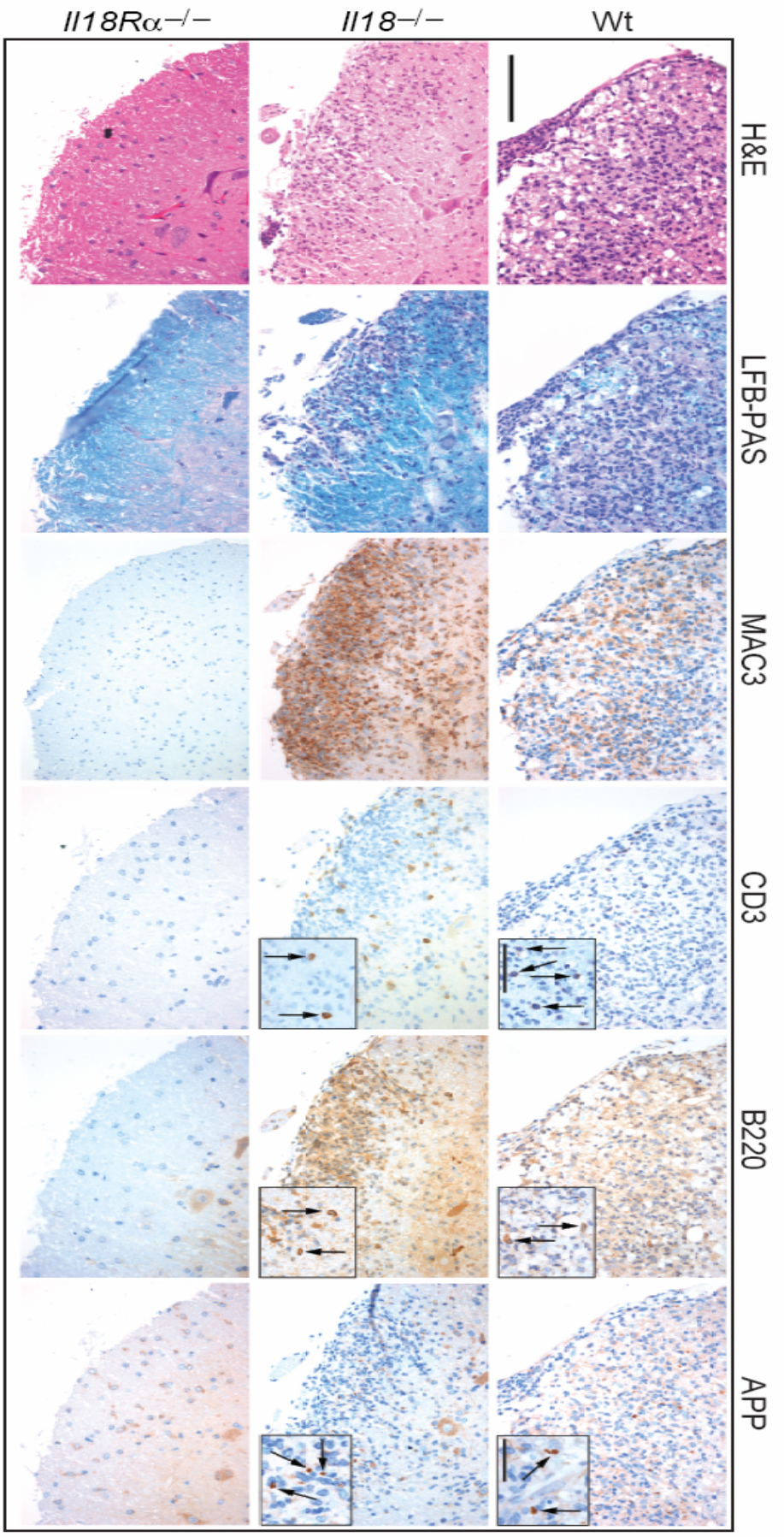


Figure 4: Discordant EAE progression in IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice. (**a**) EAE was induced in wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice by subcutaneous immunisation with MOG_{35-55}. Data display the mean EAE score of each group. Shown is one representative of 3 individual experiments. (**b**) Flow cytometry measuring expression of IL-18Rα, using an extracellular-binding anti-IL-18Rα Ab, in the blood of wt and IL-18Rα$^{-/-}$ mice. Shown is one representative of 3 individual mice.

a)



b)

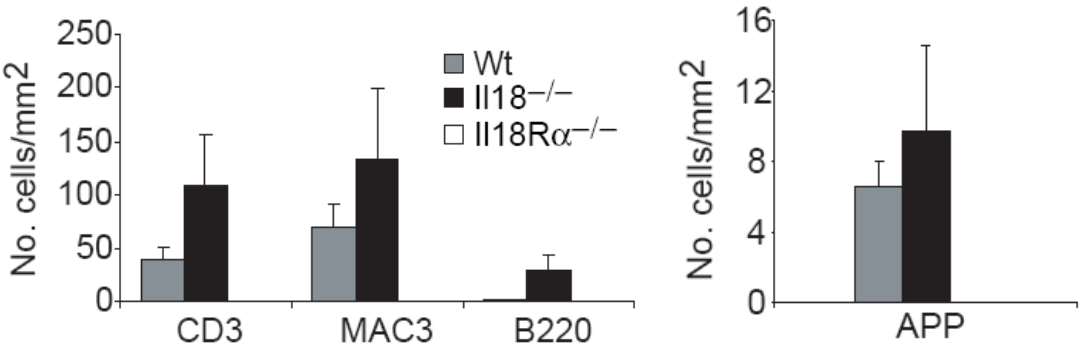


Figure 5: Histopathological analysis of IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice. (**a**) Paraffin-embedded spinal cord sections from PBS-perfused wt, IL-18$^{-/-}$, IL-18Rα$^{-/-}$ and naive wt animals on day 28 after induction of EAE with MOG_{35-55}. Haematoxylin and eosin (H&E) assesses cell infiltration, luxol fast blue (LFB) assesses demyelination and CD3^{+}, MAC3^{+} and B220 stainings demonstrate T cell, macrophage and B cell infiltration, respectively. Scale bar, 200μm. (**b**) Quantification of CD3^{+}, MAC3^{+}, B220^{+} and APP^{+} cells in histological sections of spinal cord from actively immunised wt (n=8), IL-18$^{-/-}$ (n=5) and IL-18Rα$^{-/-}$ (n=4) mice 28 days post-immunisation.

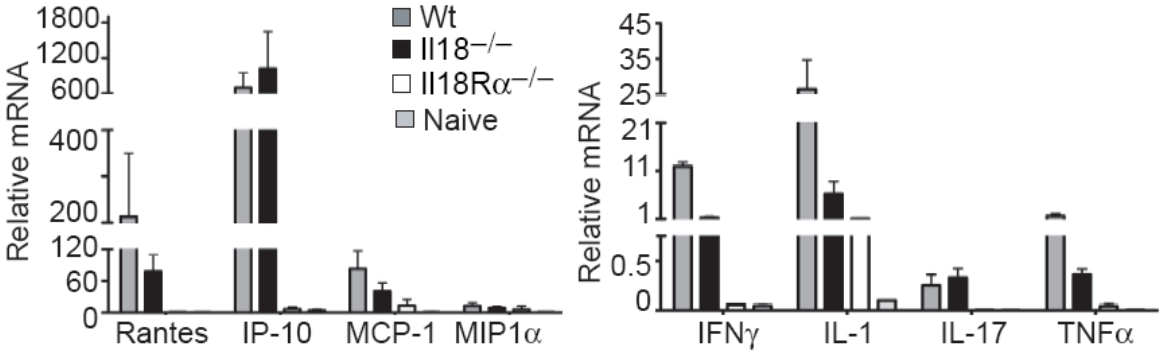


Figure 6: Analysis of cytokine and chemokine expression in the CNS of MOG_{35-55}-immunised mice. Chemokine and inflammatory cytokine mRNA expression in the spinal cords of MOG_{35-55}-immunised wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice was analysed 14 days post-immunisation by real-time PCR. Naïve spinal cord was analysed as a negative control. Error bars represent ± S.E.M. Data are representative of at least 2 individual experiments and combine 2 mice in each group.

Anti-IL-18Rα mAb-treated mice are resistant to EAE

The discordant behaviour of IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice with regards to EAE strongly points towards an additional IL-18Rα ligand with powerful encephalitogenic properties. In order to verify that IL-18Rα and IL-18 have independent biological functions, we blocked IL-18Rα in EAE-susceptible IL-18$^{-/-}$ mice. Treatment of IL-18$^{-/-}$ mice with anti-IL-18Rα monoclonal Abs (mAb), given 1 day pre-immunisation and every 3 days thereafter until the end of the experiment, significantly reduced disease development (**Fig. 7a**). Administration of anti-IL-18Rα mAbs did not lead to deletion of IL-18Rα-expressing cells nor did it alter the composition of peripheral leukocytes in the blood, LN or spleen (**Table 2**). Interestingly, treating IL-18$^{-/-}$ mice with mAbs against IL-18Rα post-immunisation (day 10 p.i.) also abrogated EAE progression (**Fig. 7b**) suggesting that IL-18Rα engagement is an important event during the effector phase of EAE. Combining the facts that IL-18Rα blockade prevents EAE even in mice in which its ligand is completely removed by gene-targeting and that IL-18 has a reportedly low affinity to IL-18Rα, we propose that another ligand must be responsible for the engagement, signalling and immune development mediated by IL-18Rα.

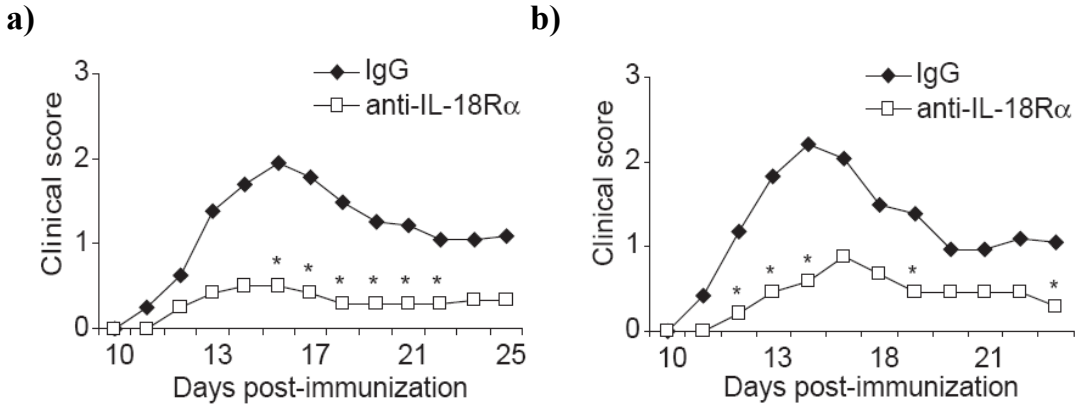


Figure 7: Anti-IL-18Rα Ab treatment abrogates EAE development and progression. (**a**) IL-18$^{-/-}$ mice were treated with 450 μg anti-IL-18Rα Ab or control IgG 1 day pre-immunisation with MOG_{35-55} and with 300 μg antibody for every 3 days thereafter. (**b**) IL-18$^{-/-}$ mice were immunised and treated with 300 μg anti-IL-18Rα Ab or control IgG at the first sign of disease. Shown are representatives of at least two individual experiments ($n \geq 5$ mice/group).

Table 2: IL-18Rα Ab treatment does not affect leukocyte composition

Immune cells	IgG (% +/- SEM)	α–IL-18Rα Ab (% +/- SEM)
CD4	35.5 +/- 2.6	38.85 +/- 4.15
CD8	22.8 +/- 1.75	25.15 +/- 0.95
B220	34.4 +/- 3.8	32.65 +/- 3.75
NK1.1	1.6 +/- 0.1	1.5 +/- 0.1
NKT	0.35 +/- 0.05	0.4 +/- 0.2
CD11b	1.0 +/- 0.1	0.7 +/- 0.0
GR1	2.65 +/- 0.35	1.85 +/- 0.05

IL-18$^{-/-}$ mice were subcutaneously immunised with MOG_{35-55} and treated with anti-IL-18Rα mAb or IgG. The composition of leukocytes in LNs, spleen and blood was analysed 7 days later by flow cytometry. Shown is the percentage of leukocytes and accessory cells in LNs, which is representative of spleen and blood.

CNS-specific IL-18Rα deletion does not affect the development of EAE

By administering anti-IL-18Rα antibodies to IL-18$^{-/-}$ mice, we were able to abrogate EAE development, even when the antibodies were given at the time of disease onset. The CNS micro-environment is critical for the ongoing inflammatory response that occurs in MS and EAE. Through the production of cytokines and chemokines by CNS-resident cells, such as microglia, immune cells are attracted into and are reactivated in the CNS. We therefore sought to determine if IL-18Rα expression on CNS-resident cells is crucial for EAE development. To do so, we generated bone marrow chimeric mice by lethally irradiating wt or IL-18Rα$^{-/-}$ recipients and reconstituting them with BM from either wt of IL-18Rα$^{-/-}$ donor mice. This produced the following groups of mice that confined IL-18Rα to either the peripheral or CNS compartment: wt → IL-18Rα$^{-/-}$ and IL-18Rα$^{-/-}$ → wt. For a positive control, wt → wt mice were also generated. Immunisation of the BM-chimeric mice demonstrated that while mice with IL-18Rα$^{-/-}$ CNS but a wt immune system are completely susceptible to EAE

(wt → IL-18Rα$^{-/-}$), expression of IL-18Rα$^{-/-}$ in the peripheral immune compartment is an absolute requirement for the development of disease (IL-18Rα$^{-/-}$ → wt) (**Fig. 8**).

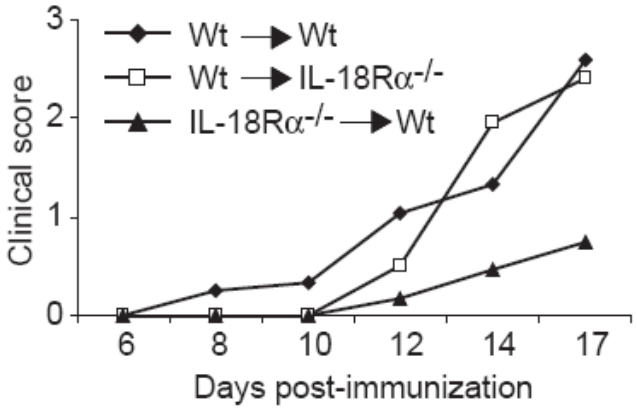


Figure 8: IL-18Rα expression in the CNS is not required for EAE pathogenesis. EAE progression in WT → WT, WT → IL-18Rα$^{-/-}$ and IL-18Rα$^{-/-}$ → WT BM-chimeric mice actively immunised with MOG_{35-55} peptide. Shown is one representative of 2 individual experiments ($n \geq 5$ mice/group).

IL-18Rα$^{-/-}$ CD4^{+} T cells are not defective in activation marker upregulation

IL-12p35$^{-/-}$ mice have a defective T_H1 response, characterised by decreased levels of IFNγ production, and are hypersusceptible to MOG_{35-55}-induced EAE. We were able to determine that MOG-reactive lymphocytes from IL-18Rα$^{-/-}$ mice were, in contrast to those from wt and IL-18$^{-/-}$ mice, also deficient in IFNγ production (**Fig. 3c**). However, in contrast to IL-12p35$^{-/-}$ mice, IL-18Rα$^{-/-}$ mice were almost completely resistant to EAE induction (**Fig. 4a**). Therefore, EAE resistance of IL-18Rα-deficient mice does not result from defective IFNγ production alone. CD4^{+} T cells are known to have a critical role in autoimmune inflammation and the expression of specific cell-surface markers by these cells can determine their ability to induce or down-regulate an autoimmune response. CD62L (L-selectin) is an integrin that

regulates the homing and entry of T cells into the LNs and, as such, the expression of CD62L is decreased from the surface of T cells during T cell activation. In contrast, other integrins such as VLA-4, which binds VCAM-1 on endothelial cells and is required for entry into the target organ, and CD162 (PSGL-1) are increased on the surface of Ag-reactive T cells. The levels of another marker, CD5, are critical for determining the responsiveness and survival of T cells after Ag stimulation. T cells with elevated levels of CD5 develop Ag-specific unresponsiveness whereas $CD5^{-/-}$ T cells are hyper-responsive leading to activation-induced cell death (AICD). Furthermore, deletion of CD5 in mice results in their protection from EAE (159). Therefore we wondered if the protection of IL-18R$\alpha^{-/-}$ mice to EAE resulted from abnormal levels of CD5 or activation marker expression on the surface of activated $CD4^+$ T cells. To do so, we harvested LN cells from KLH-immunised wt, IL-$18^{-/-}$ and IL-18R$\alpha^{-/-}$ mice 7 d.p.i. and restimulated them *in vitro* for 2 days with the cognate Ag. We then looked at the ability of these cells to express the surface markers CD62L, CD162, VLA-4 and CD5 by incubating them with Abs against these markers and analysing their expression by flow cytometry. We could show that there is no difference in the expression of these activation markers in wt, IL-$18^{-/-}$ and IL-18R$\alpha^{-/-}$ $CD4^+$ T cells and therefore defective $CD4^+$ T cell activation does not explain the resistance of IL-18R$\alpha^{-/-}$ mice to EAE (**Fig. 9**).

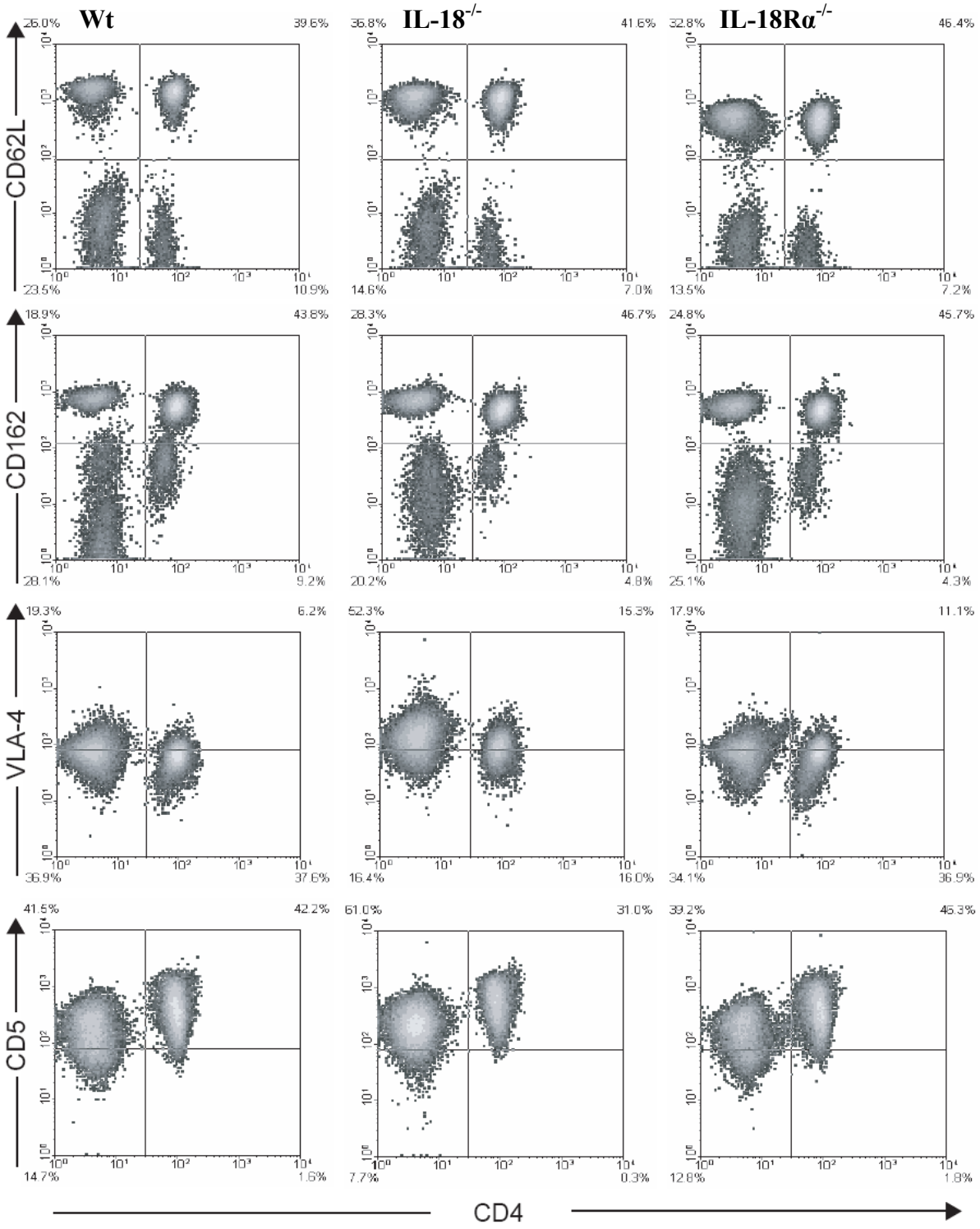


Figure 9: IL-18Rα$^{-/-}$ CD4^{+} T cells upregulate activation markers. Wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice were immunised s.c. with KLH and 7 d.p.i., LN cells were harvested and restimulated *in vitro*. After 2 days restimulation, the expression of activation markers on CD4^{+} T cells was analysed by FACS analysis.

IL-18Rα deficiency does not affect regulatory T cell numbers

Regulatory T cells have an important function in suppressing the proliferation of self-reactive T cells (50) and it has been demonstrated that the transfer of Tregs can prevent the induction of EAE in a spontaneously-occurring mouse model (160). We thus decided to analyse the effect of IL-18R-deficiency on circulating numbers of Tregs in the blood, LN and spleen. FACS analysis of these organs showed that the percentage of Tregs found in the LNs (as representative) of naïve (**Fig. 10a**) or MOG_{35-55}-immunised (**Fig. 10b**) mice does not differ between wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice.

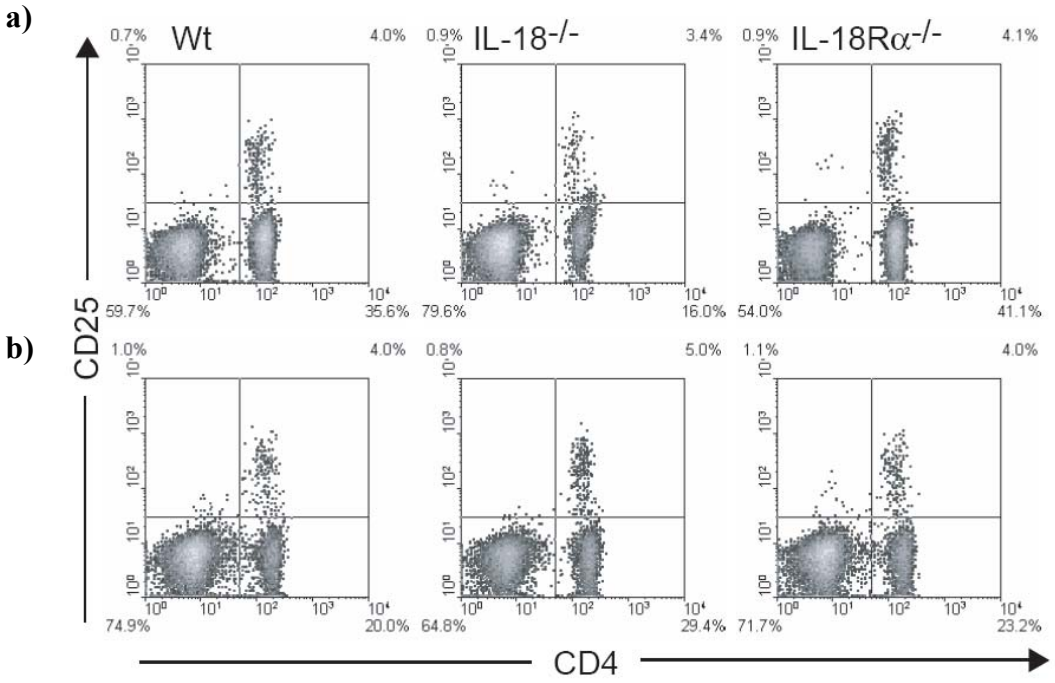


Figure 10: Regulatory T cells are not increased in EAE-resistant IL-18Rα$^{-/-}$ mice. LN, spleen and blood were isolated from naïve or MOG_{35-55}-immunised mice 14 d.p.i. and stained with anti-CD4 and anti-CD25 antibodies. The percentage of regulatory T cells was analysed by FACS analysis. Shown is the number of $CD4^{+}CD25^{+}$ T cells present in LNs, which is representative of both spleen and blood.

IL-18Rα deficiency does not affect inflammatory cell migration

EAE is characterised by a massive influx of inflammatory cells into the CNS at the peak of disease yet immune cells also invade the CNS prior to the onset of clinical symptoms (161;162). For example, recruitment of $CD4^+$ T cells into the CNS is critical for the initiation of the effector phase of EAE while invasion of polymorphonuclear leukocytes appears to have a role in orchestrating these events (163). Neutrophils constitutively express IL-18R and activation of the receptor results in enhanced adhesion molecule and chemokine expression (85). Furthermore, IL-18R expression was shown to be important for the migration of plasmacytoid DCs. Therefore to establish the effect of IL-18Rα on cellular migration, we isolated peritoneal infiltrates from wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ 24 hours after intraperitoneal injection of 3% thioglycollate and sorted for $CD11b^+$ and $GR1^+$ cells in order to obtain neutrophils. The capacity of neutrophils to migrate towards the chemoattractant fMLP was subsequently tested *in vitro*, which demonstrated that IL-$18R\alpha^{-/-}$ neutrophils migrated to the same extent as those from wt and IL-$18^{-/-}$ mice (**Fig. 11**).

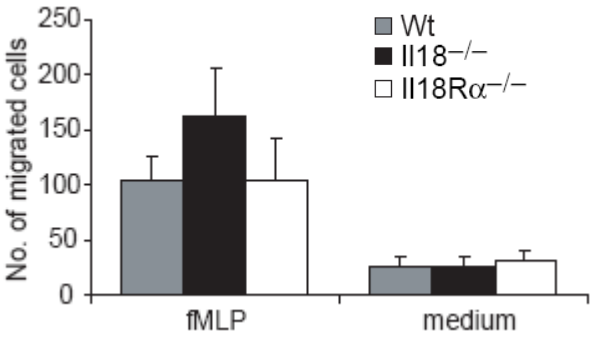


Figure 11: IL-18Rα deficiency does not affect neutrophil migration. Peritoneal neutrophils were isolated from the peritoneal exudates of wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice by cell sorting. Neutrophils were plated in an insert that was placed into a well containing either 10^{-6} M fMLP or medium. After 1 hour, the well insert was removed, fixed and the number of migrated neutrophils counted using a binocular.

IL-18Rα engagement is required for the persistence of inflammatory cells during the effector phase of disease

Although no difference in migratory capacity of neutrophils could be seen in an *in vitro* assay, we could not rule out that the IL-18Rα-deficiency affects cell migration *in vivo*. Therefore, to establish the impact of IL-18Rα on the capacity of inflammatory cells to invade the CNS at time-points of pre-clinical disease, we immunised mice and analysed the CNS for inflammatory infiltrates on day 7 post-immunisation by flow cytometry. In contrast to peak disease when the IL-18Rα$^{-/-}$ CNS is devoid of inflammatory infiltrates (**Fig. 5**), IL-18Rα$^{-/-}$ leukocytes (CD45-high cells) were capable of CNS infiltration to the same extent as those of wt and IL-18$^{-/-}$ mice at stages of pre-clinical disease (**Fig. 12a**). Detailed analysis of the invading cells showed that comparable numbers of CD4^{+} T cells, granulocytes, macrophages and B cells were present in all three strains (**Fig. 12b**). As IL-18Rα$^{-/-}$ inflammatory cells are absent from the CNS during clinical disease, it seems that IL-18Rα$^{-/-}$ inflammatory cells do not persist during the effector phase of the disease. Such results resemble those obtained with IL-23p19-deficient mice and IL-23p40-deficient mice, which are also resistant to MOG_{35-55}-induced EAE and in which inflammatory cells are found in the CNS early after immunisation but before disease begins (56).

Given the similarities between IL-18Rα$^{-/-}$ and IL-23$^{-/-}$ mice regarding their EAE resistance with concomitant inflammatory cell invasion into the CNS, we assessed the impact of IL-18Rα on IL-17 production. IL-17 producing T_H cells (T_H17) are now widely accepted to be the main pathogenic population during autoimmune inflammation (56;143). We therefore quantified the number of IL-17 producing MOG-reactive T cells invading the CNS before and after clinical disease onset in mice immunised with MOG_{35-55}. ELISpot analysis revealed that immediately prior to disease onset (9 days after immunisation), the number of MOG-specific IFNγ-secreting cells invading the CNS was similar in EAE-susceptible IL-18$^{-/-}$ and EAE-resistant IL-18Rα$^{-/-}$ mice, whereas T_H17 cells were nearly completely absent from the CNS of

IL-18Rα$^{-/-}$ mice only (**Fig 12c**). After disease onset, moreover, although we detected increased numbers of IFNγ- and IL-17-secreting cells in the susceptible IL-18$^{-/-}$ and wt mice, these cells were almost completely absent from the CNS of IL-18Rα$^{-/-}$ mice (**Fig. 12d**).

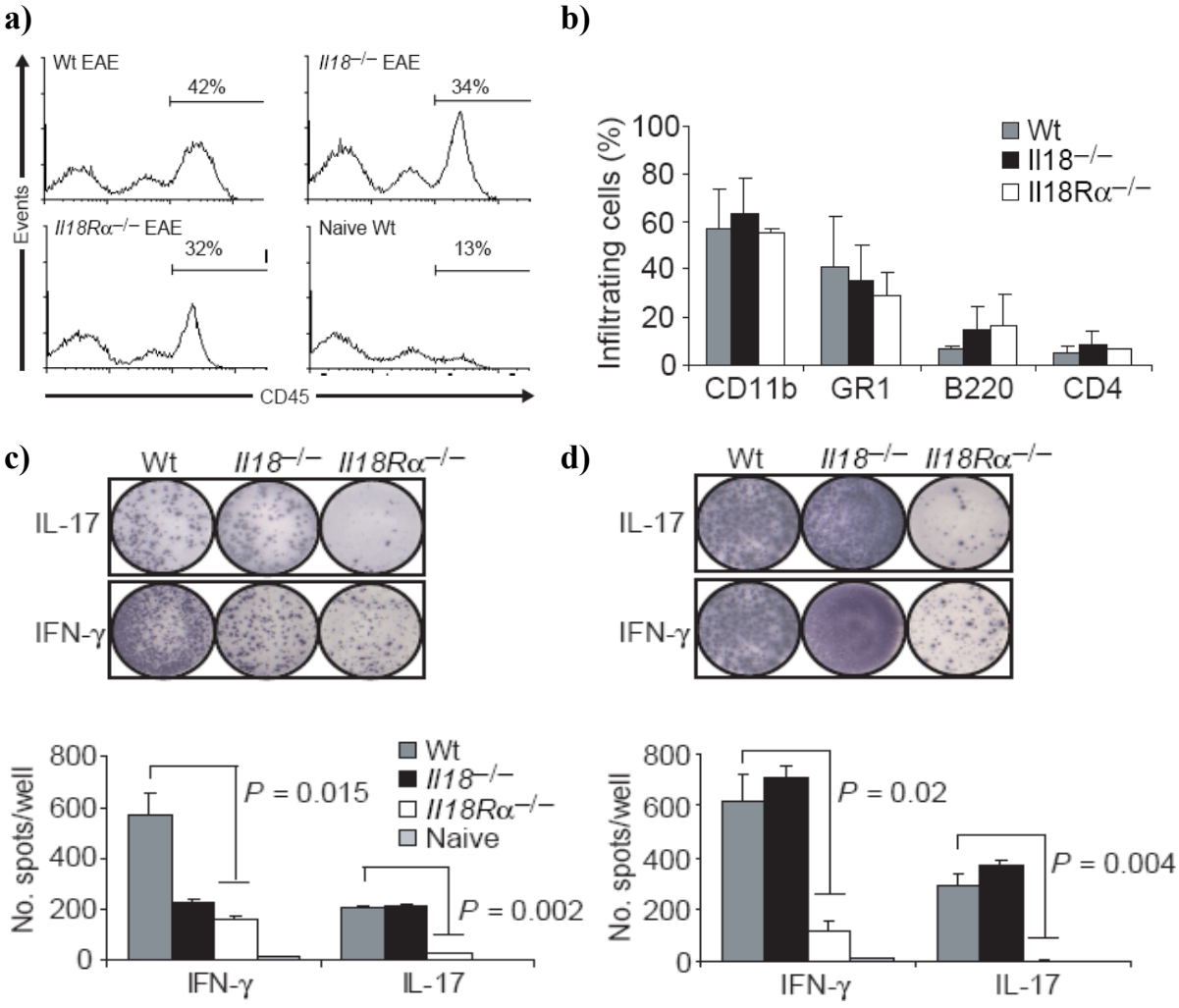
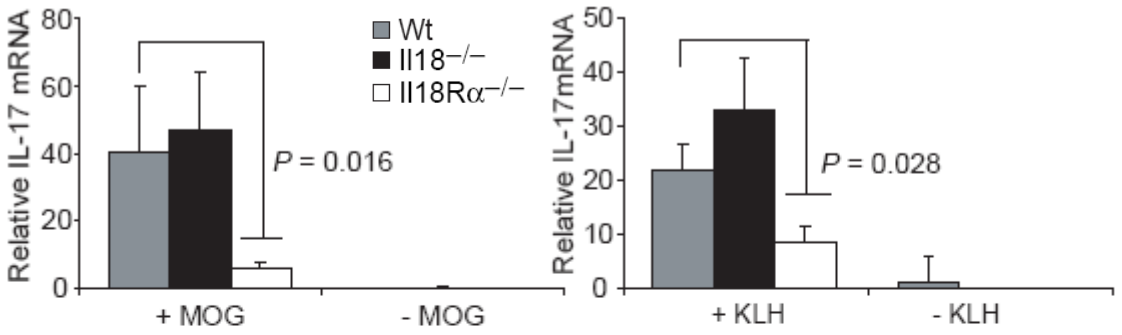


Figure 12: Tissue invasion at pre-clinical stages is not affected by IL-18Rα. (**a,b**) Flow cytometry of CNS-derived leukocytes from MOG_{35-55}-immunised wt, Il-18$^{-/-}$ and Il-18Rα$^{-/-}$ 7 days after immunisation; cells were stained with anti-CD45, anti-CD11b anti-GR1, anti-B220 and anti-CD4 antibodies. (**a**) Percent CD45^{hi} infiltrating cells in the CNS (indicated by numbers above bracketed lines). (**b**) CD45^{hi} cells were gated and percentages of infiltrating macrophages, granulocytes, B cells and CD4^{+} T cells were assessed. Naïve, non-immunised mice were used as a negative control. Shown is one representative of 3 individual experiments with 2 mice per experiment. (**c,d**) ELISpot analyses for IFNγ and IL-17 production from CNS-derived MOG-reactive lymphocytes restimulated for 18-20 hours with MOG_{35-55}. (**c**) 9 dpi pre-disease onset; (**d**) 14 dpi post-disease onset. Data in the bar graphs represent at least 2 individual experiments and combine 2 mice in each group. Shown is the mean ± S.E.M.

IL-18Rα engagement is required for T_H17 cell polarisation

To determine whether IL-18Rα$^{-/-}$ mice are defective in generating IL-17-producing T cells, we analysed IL-17 expression by freshly primed lymphocytes in vitro. We immunised wt, IL-18$^{-/-}$ as well as IL-18Rα$^{-/-}$ mice with either MOG_{35-55} peptide or KLH and collected lymphocytes from the draining LNs after 7 days. By performing real-time PCR analysis of mRNA obtained from lymphocytes after restimulation with their cognate Ag, we found that the expression of IL-17 mRNA was significantly lower in IL-18Rα$^{-/-}$ cells than in wt or IL-18$^{-/-}$ cells (**Fig 13a**). We corroborated those results by ELISA of cell culture medium of lymphocytes rechallenged with their cognate Ag *in vitro*, which demonstrated that IL-17 production was significantly decreased in IL-18Rα$^{-/-}$ but not IL-18$^{-/-}$ lymphocytes (**Fig. 13b**). In contrast to the production of IFNγ, which shows some dependence on the immunogenicity of the Ag, IL-17-secretion was consistently lower in the IL-18Rα$^{-/-}$ leukocytes, even when KLH was used as the driving Ag. Thus, the resistance of IL-18Rα$^{-/-}$ mice to EAE could certainly derive from their inability to generate sufficient numbers of inflammatory T_H17 cells.

a)



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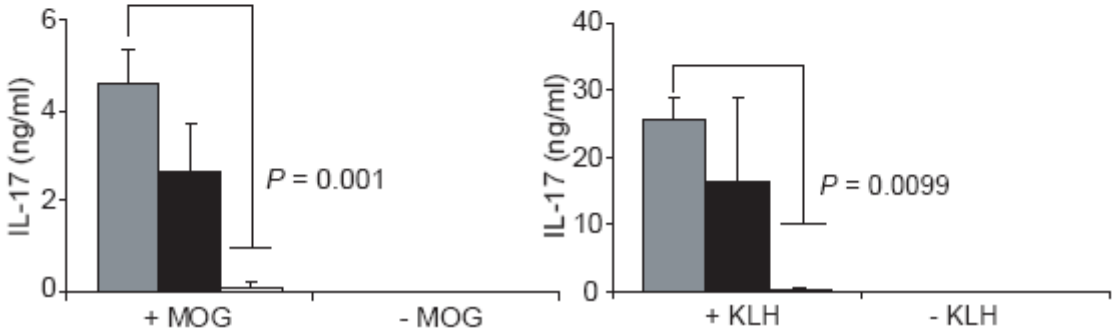


Figure 13: T_H17 induction is dependent on IL-18Rα but not IL-18. (**a**) Real-time PCR analysis of IL-17 mRNA expression by wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ lymphocytes from mice immunised 7 days previously and restimulated for 48h with 50 µg/ml MOG_{35-55} or KLH. Data are representative of 2 individual experiments combining at least 2 mice per group. Shown is the mean ± S.E.M. Results are normalized to β-actin expression and analysed in duplicate. (**b**) ELISA of IL-17 protein expression by MOG_{35-55} and KLH-restimulated lymphocytes, analysed in duplicate.

Expression of IL-18Rα on accessory cells is essential for encephalitogenicity

The results thus far show that IL-18Rα-deficiency completely prevents the development of EAE and T_H17 polarization, whereas IL-18 appears to be irrelevant for both phenotypes. The cell type on which the IL-18Rα exerts its primary effects remains unknown. This is due mainly to the fact that IL-18Rα is expressed by various cell types and tissues (83-85). In order to identify which cells are required to express IL-18Rα, we selectively expressed IL-18Rα on cells in the leukocyte compartment using irradiation bone-marrow (BM) chimeras. Following irradiation and reconstitution, the immune compartment in secondary lymphoid tissues of recipient mice is comprised of hematopoietic cells derived from donor mice (11;153). We generated BM chimeras by transferring either a 4:1 ratio of $Rag1^{-/-}$ and IL-$18R\alpha^{-/-}$ BM into wt recipients ($Rag1^{-/-}$ + IL-$18R\alpha^{-/-}$ → WT) or IL-$18R\alpha^{-/-}$ BM only into wt recipients (IL-$18R\alpha^{-/-}$ → WT); as a control, we also transferred wt BM into wt recipients (WT → WT). Because $Rag1^{-/-}$ mice do not have lymphocytes, all lymphocytes from the 4:1 $Rag1^{-/-}$ + IL-$18R\alpha^{-/-}$ → WT chimera were IL-18Rα-deficient, whereas the majority of all other non-lymphocyte leukocytes (accessory cells such as neutrophils, eosinophils, etc.) expressed IL-18Rα.

As anticipated from our previous data, IL-$18R\alpha^{-/-}$ → WT chimeric mice were resistant to EAE after immunisation with MOG peptide (**Fig. 14a**). However, addition of $Rag1^{-/-}$ BM to the IL-$18R\alpha^{-/-}$ BM ($Rag1^{-/-}$ + IL-$18R\alpha^{-/-}$ → WT) resulted in susceptibility to EAE (**Fig. 14a**). Thus, IL-18Rα expression on accessory cells is sufficient for EAE induction even when lymphocytes are IL-18Rα-deficient.

To confirm the role and function of IL-18Rα signalling on accessory cells during the effector phase of EAE, we adoptively transferred encephalitogenic MOG-reactive T cells derived from wt donor mice into both wt and IL-$18R\alpha^{-/-}$ recipient mice. Fully primed and

activated encephalitogenic T cells derived from wt mice induced EAE in wt recipient mice, yet they were incapable of inducing clinical EAE in IL-18Rα-deficient hosts (**Fig. 14b**).

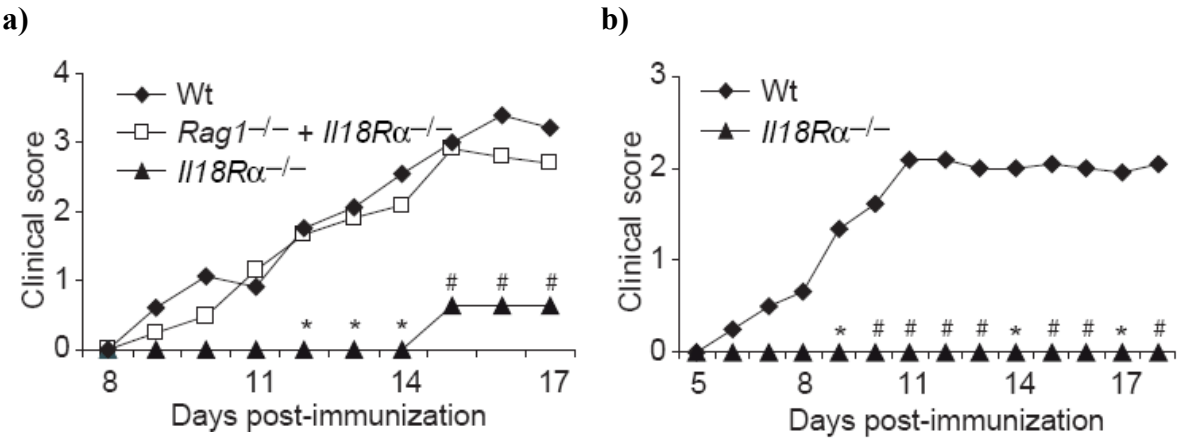


Figure 14: IL-18Rα-deficiency specifically affects non-lymphocytic leukocytes. (**a**) EAE progression in IL-18Rα$^{-/-}$ → WT, IL-18Rα$^{-/-}$ + Rag1$^{-/-}$ → WT and WT → WT BM-chimeric mice actively immunised with MOG_{35-55} peptide. Shown is one representative of 2 individual experiments ($n \geq 5$ mice/group). (**b**) EAE was induced in recipient mice by the adoptive transfer of 25 x 10^6 MOG-reactive lymphocytes into IL-18Rα$^{-/-}$ and wt mice. Shown is one representative of 2 individual experiments ($n \geq 5$ mice/group).

IL-18Rα expression on DCs is not necessary for naïve T cell priming

As IL-18Rα has been demonstrated to be present on myeloid cells (164), such as APCs which play an important role in T cell activation, we next tested the capacity of IL-18Rα-deficient APCs to prime naïve T cells. To do so, we carried out a mixed lymphocyte reaction (MLR) whereby CD4$^+$ T cells were purified from BALB/c mice and cultured for four days with wt, IL-18$^{-/-}$ or IL-18Rα$^{-/-}$ immature or mature BM-derived DCs. Measurement of their proliferation by thymidine incorporation showed no difference in the stimulatory abilities of mature (**Fig. 15a**) or immature (**Fig. 15b**) IL-18Rα$^{-/-}$ BM-DCs. Testing this in a transgenic system, we similarly purified CD4$^+$ T cells from 2d2 mice that express a transgenic TCR specific for MOG_{35-55} and co-cultured them with mature, MOG-peptide-pulsed wt, IL-18$^{-/-}$

and IL-18Rα$^{-/-}$ BM-derived DCs. Again, we found no difference in the ability of the different DCs to stimulate proliferation of the 2d2 T cells (**Fig. 15c**). To confirm this in an *in vivo* setting, we injected carboxyfluorescein diacetate succinimidyl diester (CFSE) labelled-2d2 cells into wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice, immunised them with MOG_{35-55} and analysed proliferation of 2d2 cells by flow cytometry after 4 days. Again, there was no difference in the proliferation of 2d2 cells in wt, IL-18$^{-/-}$ or IL-18Rα$^{-/-}$ mice (**Fig. 15d**). However, additional experiments using the adoptive transfer of CD4^{+} T cells expressing a transgenic TCR specific for the immunodominant epitope of OVA confirmed that IL-18Rα deficiency on non-lymphocytic leukocytes reduces the generation of T_H17 cells (**Fig. 15e**).

Despite no obvious defect in the ability of IL-18Rα$^{-/-}$ APCs to induce T cell expansion, we tested the expression of costimulatory molecules, such as CD40, CD80 and CD86, by LPS-stimulated splenic DCs. Confirming the normal T cell proliferative responses, we could detect no difference in the expression of these molecules by wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ DCs (**Fig. 16a**).

With regards to the ability of myeloid cells to reach the CNS tissue, we generated mixed BM chimeras of CD45 congenic wt and IL-18Rα$^{-/-}$ BM-donors into WT recipients and evaluated the capacity of myeloid populations to invade the CNS during disease. We first confirmed that the mice had a 1:1 ratio of WT and IL-18Rα$^{-/-}$ hematopoietic cells in peripheral blood and spleen. After EAE induction, we found both CD45.1 and CD45.2 myeloid populations in the CNS at a 1:1 ratio, indicating that there was no migratory difference between the two genotypes of myeloid cells (**Fig. 16b**).

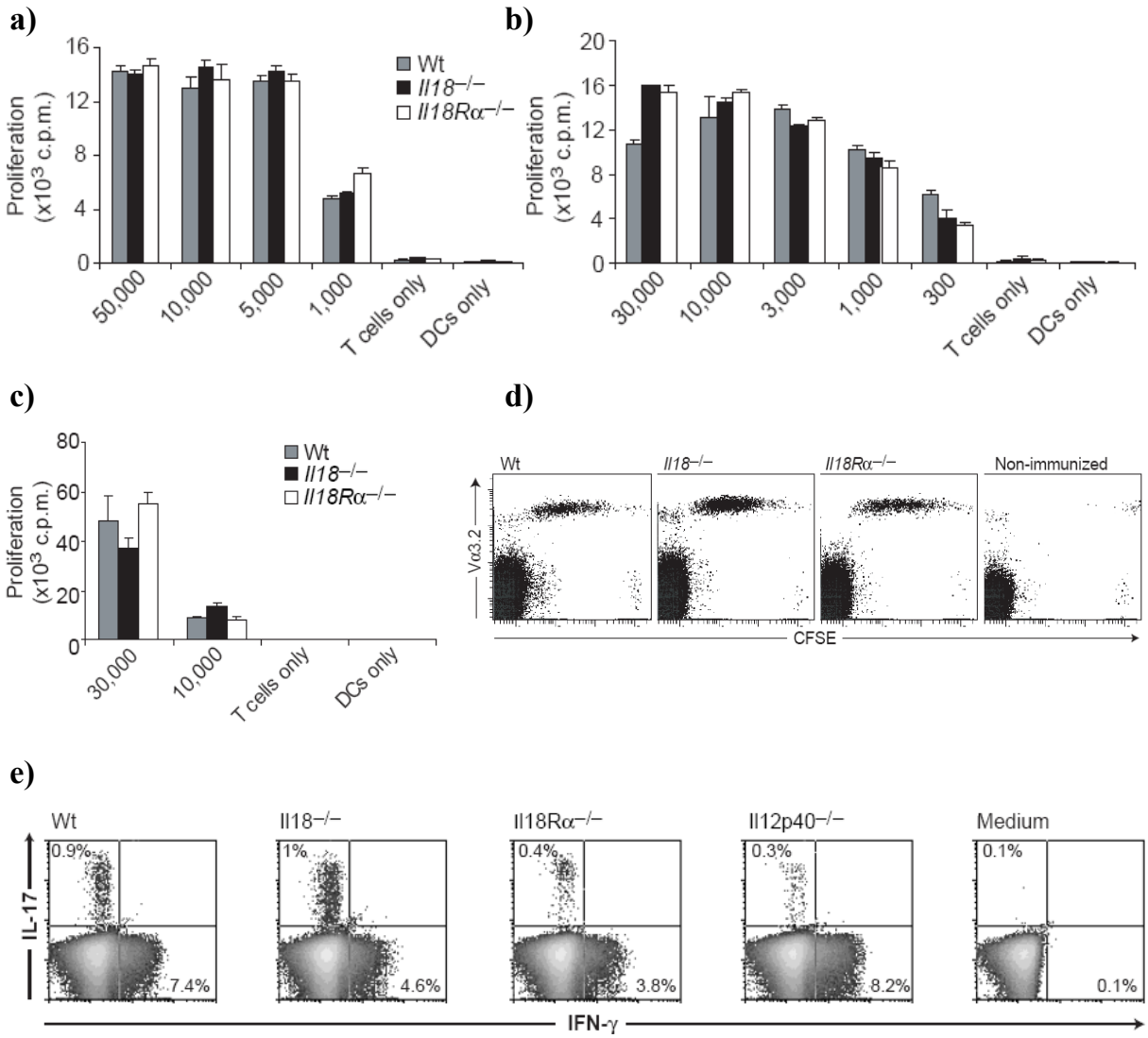
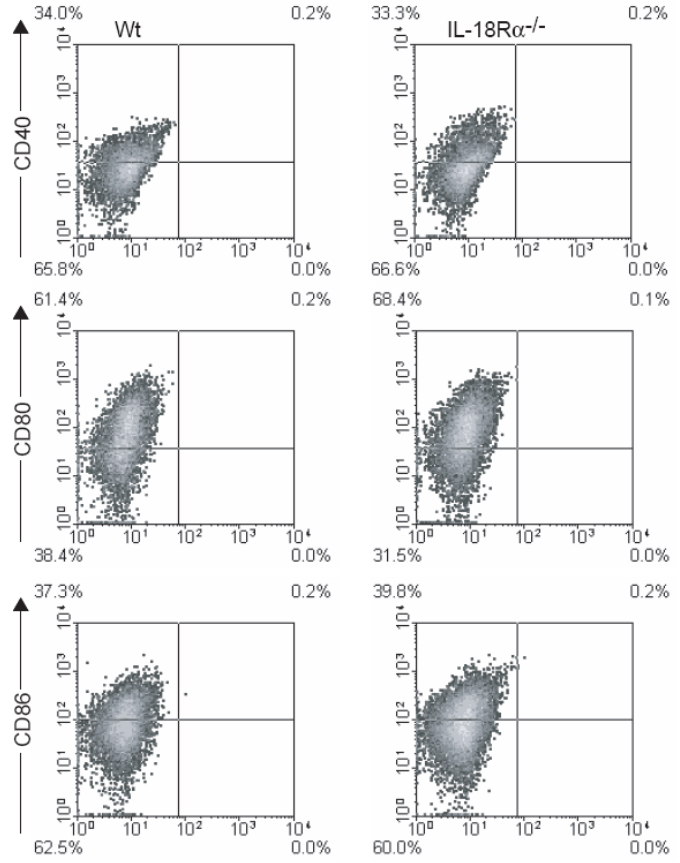


Figure 15: IL-18Rα-deficiency on accessory cells does not affect naïve T cell priming. (**a,b**) Proliferation of BALB/c $CD4^+$ T cells cultured with (**a**) mature and (**b**) immature BM-derived DCs from wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice. (**c**) Proliferation of MOG_{35-55} TCR transgenic (2d2) $CD4^+$ T cells stimulated with BM-derived DCs from wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice, matured with 10 μg/ml LPS and subsequently pulsed with 1 μg/ml MOG peptide. Error bars, S.E.M., *n*=2 mice per group. (**d**) Flow cytometry of i*n vivo* proliferation of CFSE-labelled 2d2-transferred cells into MOG_{35-55}-immunised wt, IL-$18^{-/-}$ and IL-$18R\alpha^{-/-}$ mice and a non-immunised control mouse. Data are representative of at least two experiments with at least two mice per experiment. (**e**) Flow cytometry of intracellular cytokine staining of lymphocytes from OVA-immunised mice injected with OTII splenocytes 4 days earlier. Numbers in quadrants indicate the percent of cells staining positive for the indicated cytokines.

a)



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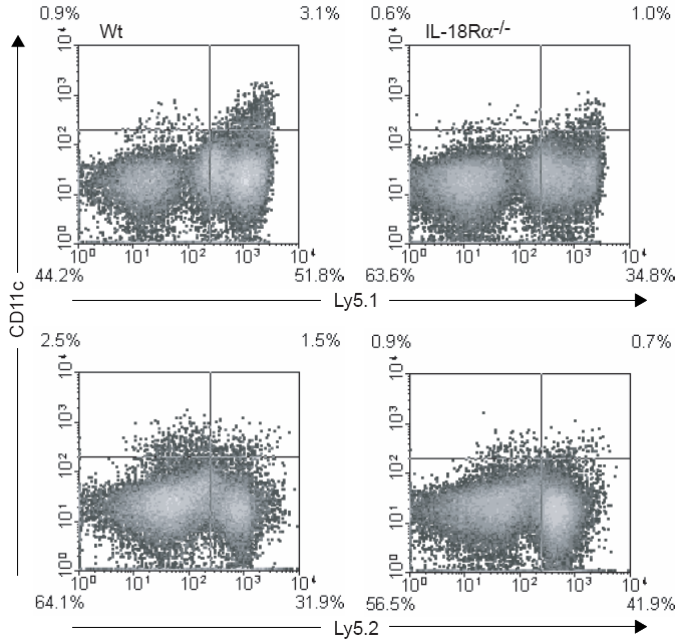

Figure 16: IL-18Rα-deficient DCs are activated and migrate to the CNS. (**a**) Flow cytometry of wt and IL-18Rα$^{-/-}$ splenocytes after overnight stimulation with LPS. Cells are stained with anti-CD40, anti-CD80 and anti-CD86 antibodies and gated on CD11c-positive cells. (**b**) Flow cytometry of CNS from CD45.1 hosts, reconstituted with a 1:1 mix of CD45.2 wt and CD45.1 wt or CD45.1 IL-18Rα$^{-/-}$ BM, 14 days post-immunisation with MOG_{35-55}. Data are representative of at least 2 experiments.

IL-18Rα-deficient APCs show limited IL-12/23p40 secretion required for pathogenic T_H17 cell generation

As restimulation with cognate Ag demonstrated a significant decrease in IL-17 production, we decided to analyse the capacity of APCs to secrete IL-12/23p40, which is necessary for the polarisation of pathogenic T_H17 cells. To determine p40 secretion, we stimulated T cell-depleted splenocytes with anti-CD40 mAb for 36 hours before analysing p40 production by ELISA. We found that in contrast to high levels of p40 production by wt and IL-$18^{-/-}$ APCs, IL-18R$\alpha^{-/-}$ APCs, like $p40^{-/-}$ APCs, showed impaired p40 production (**Fig. 17a**). Finally, we could validate the deficient IL-12/23p40 production in an adaptive immune response by restimulating lymphocytes from MOG- and KLH-immunised mice *in vitro* and analysing p40 production after 2 days. As expected, there was a significant decrease in the production of p40 by IL-18R$\alpha^{-/-}$ lymphocytes as opposed to wt and IL-$18^{-/-}$ lymphocytes (**Fig. 17b,c**). The effect of IL-18Rα on IL-23 production is specifically related to IL-23p40 upregulation as the production of IL-23p19 by MOG_{35-55}-restimulated LN cells was similar in wt, IL-$18^{-/-}$ and IL-18R$\alpha^{-/-}$ mice (**Fig. 17d**). Our data indicate that APC activation results in the production of an IL-18Rα ligand driving IL-23 secretion by APCs. Therefore the resistance of IL-18R$\alpha^{-/-}$ mice to EAE seems to result from abrogated T_H17 cell development, which is a consequence of poor p40 production by IL-18R$\alpha^{-/-}$ APCs.

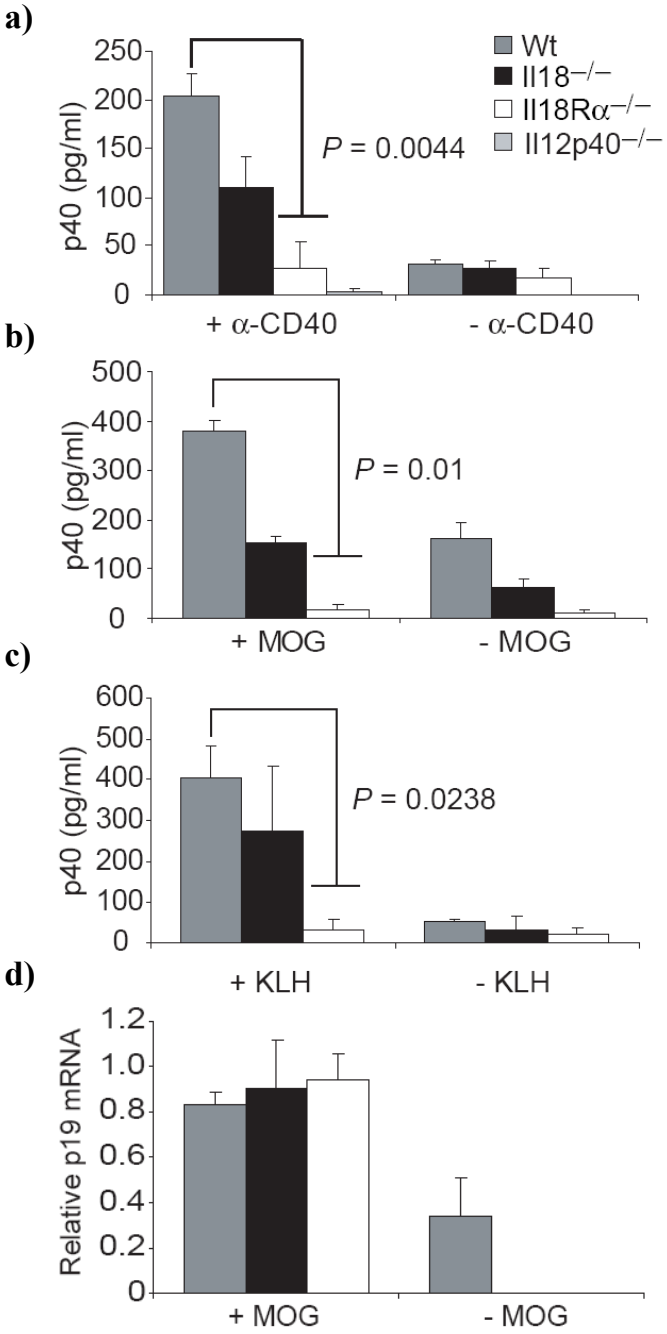

Figure 17: IL-18Rα engagement promotes the production of IL-23p40 but not IL-23p19. (**a**) ELISA of IL-12, -23 p40 production in the supernatant of T-cell depleted naive wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ splenocytes stimulated with 5 µg/ml anti-CD40 mAb for 36 hours. (**b,d**) Wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice were immunised with MOG_{35-55} in CFA and LN cells were isolated 7 days later and restimulated with 50µg/ml MOG_{35-55} or medium for 48 hours. (**b**) ELISA of p40 expression in supernatant. (**c**) ELISA of p40 expression in supernatant of LN cells from wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice immunised with KLH in CFA, isolated 7 days later and restimulated with 50µg/ml KLH or medium for 48 hours. (**d**) Real-time PCR analysis of p19 mRNA expression. Shown is the mean ± S.E.M. Data are representative of 2 individual experiments combining at least 2 mice per group.

DISCUSSION

Organ-specific inflammatory diseases generally result from inappropriate expansion and activation of effector T lymphocytes, which have escaped peripheral tolerance and react to expressed self-antigens. Until recently, T_H1 lymphocytes were thought to represent the auto-reactive T cells responsible for inducing cellular autoimmunity (92;93;165-167). However, mounting evidence indicates that T_H17 cells, and not T_H1 cells, are the pathogenic T cell effectors in autoimmunity and that their development is negatively regulated by both T_H1 and T_H2 cytokines (51;56;127;129;132;143;168).

IL-18 is not required for EAE pathogenesis

We were interested in characterising the role of IL-18 in EAE. IL-18 synergises with IL-12 in the differentiation of naive T_H cells into T_H1 cells. Contrary to initial findings, confused by the use of IL-12p40 as a target, it is now accepted that IL-12 is not pathogenic during autoimmune inflammation. Our initial goal was then to determine if EAE susceptibility of IL-12-deficient mice occurs as a result of redundancy, whereby the continued presence and activity of IL-18 in these mice is sufficient for EAE induction. Our data challenge that hypothesis as IL-12p35$^{-/-}$IL-18$^{-/-}$ double deficient mice were fully susceptible to EAE (**Fig. 1a**). We also found that single-mutant IL-18$^{-/-}$ mice are susceptible to EAE and are not defective in IFNγ production during cognate Ag recall responses (**Fig. 1b** and **Fig. 3**). These results contrast with previously published data showing that IL-18$^{-/-}$ mice are resistant to EAE (84) and are deficient in mounting auto-reactive T_H1 responses (84). The defective T_H1 cell response was suggested to result at least in part from abrogated IFNγ production by NK cells, as the transfer of NK cells from recombination activating gene (RAG)-deficient, but not from IFNγ$^{-/-}$, mice was able to rescue the susceptibility of these mice to EAE. As we could clearly demonstrate that the mice we used are deficient in IL-18 (**Fig. 2**), the discrepancy between our data and the earlier study could be explained by a different health status of the mice or their

degree of backcrossing onto the C57BL/6 background. Our results with the IL-18$^{-/-}$ mice are also in agreement with subsequent data with independently derived IL-18$^{-/-}$ mice that show susceptibility to experimental autoimmune uveitis (EAU) after using a similar methodology as was used in our study (169;170). Thus, IL-18$^{-/-}$ mice are susceptible to EAE and EAU by the standard methodology of inducing experimental autoimmunity. Other more recent reports also support our findings for a non-pathogenic role for IL-18 in autoimmunity. Santos et al demonstrated that IL-18 is unnecessary during Ag-induced T_H1 responses and is redundant in an Ag-induced arthritis model (157), which is in contrast to previous findings showing that IL-18 is required for the induction of CIA. They suggest that differences in the pathogenic mechanisms of these models or genetic background of the mice could explain the conflicting results. Meanwhile, another group has shown that IL-18 is unnecessary for the development of clinical symptoms in experimental autoimmune neuritis (EAN), which contradicts previous results demonstrating that an antibody against IL-18 ameliorates the clinical symptoms of EAN (171;172). Therefore, there is an emerging non-pathogenic role for IL-18 in autoimmunity, which is in line with both our results and the changing Th1/Th2 paradigm.

IL-18Rα engagement is crucial for EAE pathogenesis

Despite the susceptibility of IL-18$^{-/-}$ mice, we found that IL-18Rα$^{-/-}$ mice are resistant to EAE (**Fig. 4a**), which implies that an IL-18Rα-binding ligand other than IL-18 has encephalitogenic properties. The affinity of IL-18 to IL-18Rα is known to be weak and requires the presence of IL-18Rβ for increased affinity and signalling. In addition, there are a number of orphan receptors within the IL-1R superfamily and given the fact that this family of receptor subunits form heterodimers with one another (173), it is possible that IL-18Rα not only has different binding partners but also different ligands. We demonstrated the importance of IL-18Rα by significantly attenuating disease development in IL-18$^{-/-}$ mice using IL-18Rα antibodies (**Fig. 7**). Given that the known IL-18Rα-ligand, IL-18, was not present in these

mice, that result provides persuasive evidence for the existence of an alternative IL-18Rα ligand. At present we are in the process of searching for such an IL-18Rα-binding ligand as well as the composition of the ligand's full receptor complex.

Strengthening the findings that IL-18Rα is capable of interacting with a ligand other than IL-18, Lewis & Dinarello recently showed opposite responses in the rejection of IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ pancreatic islets (174), which led the authors to propose the existence of an IL-18-independent inhibitory pathway that converges with the pro-inflammatory IL-18 signalling pathway at the IL-18R. Furthermore, they suggest the involvement of IL-1F7, a member of the IL-1 family which binds IL-18 binding protein (IL-18BP) and IL-18Rα thereby inhibiting IL-18 activity. Their postulation of an additional inhibitory pathway differs from our hypothesis of an alternative, stimulatory ligand. This is probably due to their assessment of the roles of IL-18 and its receptor on pancreatic islets in mice that still have an intact immune system. Nevertheless, the generation of different phenotypes resulting from deletion of IL-18 or IL-18Rα is apparent in this model.

IL-18Rα expression in the periphery, but not the CNS, is required for EAE

One of our goals was to determine the mechanism of action of IL-18Rα activation and signalling during EAE. The development of EAE relies upon initial activation of CD4^{+} T cells in the peripheral immune system but the CNS environment is important for reactivating infiltrating cells during the effector stages of disease. In contrast to wt and IL-18$^{-/-}$ mice, the spinal cord of EAE-resistant IL-18Rα$^{-/-}$ mice is completely devoid of leukocyte infiltration at the peak time of clinical EAE (**Fig. 5**). Furthermore, there was no upregulation of inflammatory cytokines and chemokines in the CNS of IL-18Rα$^{-/-}$ mice during peak disease, which is characteristic of this time-point and a requirement for ongoing inflammation (**Fig. 6**). However, the lack of IL-18Rα in the CNS does not contribute to these findings and has no

role in the pathogenesis of EAE as mice in which IL-18Rα was deleted from the CNS only (Wt → IL-18Rα$^{-/-}$) were not protected from disease (**Fig. 8**).

On the other hand, absence of IL-18Rα from the peripheral immune compartment prevents the development of EAE as demonstrated by the resistance of IL-18Rα → wt mice to disease induction (**Fig. 8**). Therefore the presence of IL-18Rα on leukocytes is clearly important for the development of EAE. Upon analysis of T cell function, we discovered that IL-18Rα$^{-/-}$ lymphocytes are deficient in producing IFNγ in a MOG recall response (**Fig. 3c**). Nevertheless, we speculated that this could not explain the resistance of IL-18Rα$^{-/-}$ mice to EAE because mice deficient in or lacking IFNγ, such as IFNγ$^{-/-}$ or IL-12p35$^{-/-}$ mice respectively, are usually hyper-susceptible to EAE (96;129;175).

Activation of MOG-reactive CD4^{+} T cells results in the upregulation of costimulatory molecules and cytokines that are critical for the induction of EAE. During activation, the integrins L-selectin and VLA-4 are down- and up-regulated, respectively, which diverts the homing of encephalitogenic T cells from the LNs to the CNS. In addition, the levels of T cell CD5 expression critically determine the degree of T cell responsiveness. Deletion or block of CD5 and VLA-4, respectively, has already been demonstrated to induce EAE resistance in mice (159;176). In the case of IL-18Rα deficiency, however, alterations in expression of these activation markers did not lead to EAE resistance as there was no difference in their expression levels on CD4^{+} T cells from wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice (**Fig. 9**).

Finally, EAE resistance of IL-18Rα$^{-/-}$ mice did not result from an increase in the number of immunosuppressive regulatory T cells in the LNs, spleen or blood of these animals (**Fig. 10**).

IL-18Rα deficiency does not affect leukocyte migration

The development of EAE in wt mice is dependent on the infiltration of activated $CD4^+$ T cells into the CNS, an event that is accompanied by the influx of other immune cells including B cells, macrophages and granulocytes (161-163). In fact, we could show that at early time-points of disease, the CNS is predominantly infiltrated by granulocytes and macrophages (**Fig. 12b**). The activity and migration of granulocytes into the CNS are critical for EAE development and disruption of these cells can abrogate the effector phase of EAE (163). We tested whether the absence of immune cells in the IL-18Rα$^{-/-}$ CNS was due to a defect in the migration properties of granulocytes. However, the *in vitro* migration of neutrophils from IL-18Rα$^{-/-}$ mice was identical to that from wt and IL-18$^{-/-}$ mice (**Fig. 11**). Surprisingly, although inflammatory cells are absent from IL-18Rα$^{-/-}$ spinal cords at the peak time of clinical EAE, we could show that comparable *in vivo* granulocyte infiltration occurs in all three groups of mice prior to disease onset (**Fig. 12b**). Interestingly, not only were granulocytes present in the IL-18Rα$^{-/-}$ CNS, but similar numbers of other CNS-invading leukocytes, including $CD4^+$ T cells, could also be detected at this time-point (**Fig. 12a,b**). Therefore, IL-18Rα-deficiency does not affect the initial migratory properties of leukocytes but their persistence in the CNS.

IL-18Rα deficiency indirectly affects IL-17 production

The presence of inflammatory infiltrates early on in the IL-18Rα$^{-/-}$ CNS is similar to the phenotype observed in IL-23$^{-/-}$ mice (177), whose resistance to EAE likely results from their inability to expand and maintain IL-17-producing T_H cells (53;56;178;179). Similar to these findings, and despite comparable $CD4^+$ T cell infiltration, IL-18Rα$^{-/-}$ mice showed a significant decrease in T_H17 cells in the CNS on day 7 post-immunisation (**Fig. 12c**). Furthermore, IL-18Rα$^{-/-}$ LN-derived lymphocytes were unable to produce IL-17 in response to cognate Ag restimulation, in contrast to wt and IL-18$^{-/-}$ lymphocytes (**Fig. 13**). Therefore the IL-18Rα deficiency affects the production of the novel encephalitogenic cytokine IL-17.

Previous data has shown that the expression of IL-18Rα is broadly distributed and not confined to NK cells and T cells as previously thought (83-85). By performing experiments using BM chimeras, we demonstrated that the loss of IL-18Rα from an accessory cell, and not from T or B cells, was responsible for the loss of T_H17 cells and for EAE resistance (**Fig. 14a**). The importance of IL-18Rα on accessory cells was emphasized by adoptive transfer of otherwise encephalitogenic wt T cells that did not induce EAE in IL-18Rα$^{-/-}$ mice (**Fig. 14 b**). Again, this result is similar to IL-12p40$^{-/-}$ mice, which showed decreased severity of EAE upon transfer of encephalitogenic wt T cells (145).

IL-18Rα expression on APCs is required for IL-23 production

The interaction of APCs with T cells and the concomitant generation of costimulatory signals and cytokines is an absolute requirement for T cell proliferation and polarisation. Using *in vitro* simulation of either allogeneic or transgenic T cells by IL-18$^{-/-}$ or IL-18Rα$^{-/-}$ BM-DCs, we found that T cell activation and expansion are not affected by the loss of IL-18Rα (**Fig. 15a,b,c**). Indeed, adoptively transferred naïve TcR-Tg lymphocytes proliferated to the same extent in immunised IL-18Rα$^{-/-}$ hosts as in wt and IL-18$^{-/-}$ ones (**Fig. 15d**). Normal proliferation of T cells coincided with similar expression of costimulatory molecules by wt, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ splenic DCs (**Fig. 16a**). Despite adequate proliferation, adoptively transferred Tg lymphocytes were incapable of upregulating IL-17 production in IL-18Rα$^{-/-}$ hosts. These results were similar to those obtained in IL-12p40$^{-/-}$ hosts, but in contrast to wt and IL-18$^{-/-}$ mice (**Fig. 15e**). Finally, we demonstrated that IL-18Rα signalling on APCs is critical for the secretion of IL-23p40, but not IL-23p19, and the subsequent maintenance of IL-17-secreting T cells (**Fig. 17**). Our data thus support the hypothesis that IL-17 secretion is dependent on the continuous support of T_H17-promoting APCs.

Conclusion

Using the animal model of MS, we have provided additional evidence against the T_H1 paradigm of autoimmunity by demonstrating a non-pathogenic role for IL-18. In contrast, however, we show that IL-18Rα is essential for the development of EAE, thus implying the presence of an alternative IL-18Rα-binding ligand. Furthermore, activation of IL-18Rα is necessary for the expansion or survival of T_H17 cells in an IL-23-dependent manner. In a simplified scheme, the production of the alternative IL-18Rα-binding ligand during Ag recognition leads to IL-18Rα signalling, on the surface of an APC, and subsequent IL-23 release. However, the precise cellular location of IL-18Rα activation during EAE is still unknown and a much more complex and indirect pathway possibly exists for its role in IL-17 production. Nevertheless, the identification of the alternative IL-18Rα-binding ligand could provide a potent novel therapeutic approach for the treatment of organ-specific inflammatory diseases such as MS. The attractive feature of this IL-18Rα-dependent pathway for therapeutic targeting is that its loss does not completely suppress immunity, but rather abrogates the development of pathogenic autoimmune effector T cells.

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EDUCATION

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09.1998-07.2002	University of Manchester, England BSc. Biomedical Sciences with Industrial Experience Degree classification: First Class Honours
07.2000-09.2001	Aventis Pharma, Frankfurt, Germany Industrial experience research year in the Cardiovascular Disease Group.
10.1997-07.1998	University of Siena, Italy Study of Italian language and culture
09.1990-07.1997	European School, Luxembourg Awarded European Baccalaureate

HONOURS & AWARDS

01.2004-01.2006	Meyerhof fellowship awarded by the Neuroscience Center Zurich
03.2002	Awarded Novartis Research Prize for best Industrial Placement Project

PUBLICATIONS

- Gutcher I. & Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J. Clin. Invest*., 117(5):1119-1127

- Gutcher I., Urich E., Wolter K., Prinz M. & Becher B. (2006). IL-18-independent engagement of IL-18Rα is required for the development of autoimmune inflammation. *Nat. Immunol.*, 7(9): 946-953

- Urich E., Gutcher I., Prinz M. & Becher B. (2006). Autoantibody-mediated demyelination depends on complement activation but not activatory Fc-receptors. *Proc. Natl. Acad Sci. USA*, 103(49): 18697-702

- Prinz M., Garbe F., Schmidt H., Mildner A., Gutcher I., Piesche M., Schroers R., Weiss E., Kirschning C.J., Rochford C.D.P., Brück W. & Becher B. (2006). Innate Immunity mediated by TLR9 modulates pathogenicity in an animal model of multiple sclerosis. *J. Clin. Invest.*, 116(2): 456-464

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- Bachmann A., Gutcher I., Kopp K., Brendel J., Bosch R.F., Busch A.E. & Gogelein H. (2001). Characterization of a novel Kv1.5 channel blocker in Xenopus oocytes, CHO cells, human and rat cardiomyocytes. *Naunyn Schmiedebergs Arch Pharmacol.,* 364(5): 472-478

PATENTS

Methods for treating autoimmune or demyelinating diseases: EP 06/110301

CONGRESSES ATTENDED

10.2006	International Congress of Neuroimmunology, Nagoya, Japan. Oral presentation and poster
03.2006	Swiss Allergology and Immunology Congress, Zurich. Oral presentation.
10.2005	ZNZ (Neuroscience Center Zurich) Symposium, Zurich. Poster presentation
03.2005	NCCR (National Center for Competence in Research) Symposium, Ittingen. Poster presentation & data blitz.
03.2005	4th day of Clinical Research, University Hospital Zurich. Poster presentation
02.2005	Neuroimmunologische Arbeitsgruppe, Seeon. Oral presentation
10.2004	ZNZ Symposium, Zurich. Poster presentation
07.2004	12th International Congress of Immunology and 4th Annual Conference of FOCIS, Montreal. Poster presentation
05.2004	ZNZ PhD retreat, Valens. Oral presentation
03.2004	NCCR Symposium, Konstanz. Poster presentation